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Novel Separation Techniques Employing Subcritical Water and Supercritical Carbon Dioxide

by

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Abstract

This thesis describes the development of novel separation techniques employing water and carbon dioxide in order to reduce the consumption of costly, toxic and environmentally hazardous solvents often employed in high performance liquid chromatography (HPLC). One such developed system employs CO_2 as a non-polar modifier to the water mobile phase of subcritical water chromatography (SWC), which significantly increases the solvating power of the mobile phase and reduces the operating temperature of the system. This improvement in solvating power is demonstrated by the separation of a variety of analytes which have previously been challenging or not possible to analyze by conventional SWC. Further, since both water and CO_2 are inexpensive, safe, environmentally friendly, and compatible with the flame ionization detector (FID), this system is a green alternative to conventional HPLC which allows for the use of the universal and inexpensive FID. This technique is applied to the separation of a pharmaceutically relevant analyte and the use of ultrasound to promote better mixing of the mobile phase components is also explored. Overall, this developed method considerably extends the range of non-polar analytes amenable to SWC analysis, while maintaining the beneficial conventional SWC features of FID use and environmental compatibility.

A second separation technique has also been developed which takes advantage of the limited miscibility of water and CO_2. This reduced solubility allows for conditions to be attained that create a stationary phase of water lining the inside of an uncoated stainless steel capillary where separations can be performed using a supercritical CO_2

mobile phase and a water stationary phase. This system offers very good sample capacity, peak symmetry, retention time reproducibility, and often little need for sample preparation. Also, perhaps for the first time, this separation system employs an entirely green chromatographic stationary phase and mobile phase while preserving FID utility. Analytes such as alcohols, carboxylic acids, phenols, and tocopherols are employed to investigate this relatively inexpensive and robust method. As an application, the system is used to analyse non-ionic surfactants, ethanol in alcoholic beverages and biofuel, and caffeine levels in drinks. Further, since compounds are easily dissolved in the water phase, the effect of adding these compounds to the stationary phase as modifiers is also explored.

Acknowledgements

I owe many thanks to Professor Kevin Thurbide for his guidance, support and encouragement. I could not have had a better supervisor and mentor for my journey through graduate studies. Also, I would like to acknowledge the members of the Thurbide research group for their helpful assistance and for providing a wonderful working environment, thank you all. Finally, I would like to thank Holly, my parents, my friends, and my family for their support and encouragement over the years.

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List of Symbols, Abbreviations, and Nomenclature

AB	Alberta
~	Approximately
Arg	Arginine
AZ	Arizona
Asp	Asparagine
atm	Atmosphere (1 atm = 101325 Pa)
Ave	Average
BTEX	Benzene, toluene, ethylbenzene and xylene
×	By
CO_2	Carbon dioxide
CS_2	Carbon disulfide
cm	Centimetre
CCC	Counter-current chromatography
°C	Degrees Celsius
°C/min	Degrees Celsius per minute
DMSO	Dimethyl sulfoxide
DBS	Dodecylbenzylsulfonic acid
C8	Eight carbon chain
C18	Eighteen carbon chain
ESI-MS	Electrospray ionization mass spectrometry
E10	Ethanol, 10 % (v/v)

e.g.	Exempli gratia (for example)
FID	Flame ionization detector
C4	Four carbon chain
GC	Gas chromatography
GHz	Gigahertz
Gly	Glycine
g/mol	Grams per mole
>	Greater-than
≥	Greater-than or equal to
HPLC	High performance liquid chromatography
HTLC	High temperature liquid chromatography
His	Histidine
HCl	Hydrogen chloride
HILIC	Hydrophilic interaction liquid chromatography
i.e.	id est (in other words)
IL	Illinois
in	Inch
IN	Indiana
I.D.	Inside diameter
Ile	Isoleucine
kg	Kilogram
kHz	Kilohertz
<	Less-than

L	Litre
MA	Massachusetts
MΩ cm	Megaohm centimetres (1 MΩ cm = 10 000 S/m)
MPa	Megapascal
Met	Methionine
m	Metre
µg	Microgram
µL	Microlitre
µL/min	Microlitres per minute
µm	Micrometre
mg/mL	Milligrams per millilitre
mL	Millilitre
mL/min	Millilitres per minute
mm	Millimetre
MN	Minnesota
min	Minute
MW	Molecular weight
M	Moles per litre
mol/L	Moles per litre
nm	Nanometre
NE	Nebraska
pK_a	Negative logarithm of the acid disassociation constant
pH	Negative logarithm of the hydronium concentration

NV	Nevada
NH	New Hampshire
n-alcohol	Normal alcohol
n	Number of iterations
ODS	Octadecyl-bonded silica
C_1-C_5	One carbon to five carbon
ON	Ontario
O.D.	Outside diameter
pcHPLC	Packed capillary high performance liquid chromatography
Pa	Pascal
%	Percent
%RSD	Percent relative standard deviation
Phe	Phenylalanine
PBD	Polybutadiene
PEEK	Polyether ether ketone
PEG	Polyethylene glycol
PRP	Polymeric reversed phase
KNO_3	Potassium nitrate
psi	Pounds per square inch (1 psi = 6895 Pa)
Pro	Proline
QC	Quebec
®	Registered trademark
S/m	Siemens per metre

$AgNO_3$	Silver nitrate
NaOH	Sodium hydroxide
SWC	Subcritical water chromatography
SFC	Supercritical fluid chromatography
TFA	Trifluoroacetic acid
Tyr	Tyrosine
UHPLC	Ultrahigh pressure liquid chromatography
UV-Vis	Ultraviolet-visible spectroscopy
USA	United States of America
Val	Valine
v/v	Volume per volume
WA	Washington
W	Watt
W/cm^3	Watts per cubic centimetre
ZDV	Zero dead volume

Chapter One: **INTRODUCTION**

High Performance Liquid Chromatography (HPLC) is one of the most commonly employed separation techniques. Specifically, it is a technique widely used for biochemical, biomedical, pharmaceutical and environmental analysis.[1] Currently, the majority of the HPLC separation systems in use employ 4.6 mm I.D. columns which require mobile phase flow rates of approximately 1 mL/min or higher to operate efficiently. As such, the consumption of solvents commonly used as HPLC mobile phase components poses a significant economic impact to those employing this separation technique. The cost incurred when using these solvents can be further exaggerated in the event of an unexpected shortage. One such shortage occurred for acetonitrile in 2008 which was caused by multiple factory shutdowns due to environmental concerns, hurricane damage, and the global economic slowdown of 2008-2011. The great acetonitrile shortage of 2008 caused a tripling of the price which imposed a significant increase of cost for those who employ HPLC.[2]

In addition to the economic concerns, many of the most frequently employed mobile phase solvents are toxic, which can cause health risks to the operator, and the production, disposal, and accidental release of these solvents can have a serious negative impact on the environment. In this regard, reducing the consumption of these commonly employed HPLC solvents can have a great positive benefit, both economically and environmentally. The main theme of my research is to advance the development of separation techniques employing alternate solvent systems in order to reduce the consumption of conventional HPLC solvents.

1.1 Reduced-Scale Liquid Chromatography

Historically, in order to reduce solvent consumption, the size of separation column has been scaled down. As demonstrated in Table 1-1, the optimal mobile phase flow rate is roughly proportional the cross-sectional area of the separation column (i.e. the square of the diameter).[3] Therefore, a reduction of the column I.D. by a factor of two results in an approximate four-fold decrease in optimal flow rate and, in turn, a four-fold reduction in the consumption of mobile phase solvents.

Table 1-1: Diameter, optimal flow rates and approximate sample loading for commonly available commercial HPLC columns.

Column Description	Inner Diameter (mm)	Optimal Flow Rate (µL/min)	Sample Load (µg)
Analytical	4.6	500-3000	100-1500
Narrowbore	2.1	150-500	50-120
Microbore	1.0	10-100	10-50
Capillary	0.3	1-10	1-10

1.1.1 Packed capillary HPLC

As research into smaller diameter columns progressed, the use of fused silica tubing as column housings became prevalent due to its availability and its mature state of development stemming from research into open tubular gas chromatography (GC). The use of these capillaries, packed with conventional HPLC stationary phase particles (on the order of 3 to 5 µm in diameter), resulted in columns becoming available in the 0.3 mm I.D. range. The use of these packed capillary high performance liquid

chromatography (pcHPLC) columns resulted in the reduction of mobile phase flow rates by multiple orders of magnitude when compared to 4.6 mm I.D. columns. In addition to the reduction in mobile phase flow rate, capillary columns have additional advantages over analytical columns.

One such advantage is the improvement in chromatographic efficiency as the column diameter decreases. This observed improvement in efficiency has been attributed to reduction of both the A-term (eddy diffusion) and the C-term (resistance to mass transfer) contributions to the van Deemter equation.[4] The reduction in A-term contribution stems from more ordered packing of the particle bed in smaller-diameter columns while the reduction in C-term was partially explained by differences in solvent viscosities resulting from changes in back pressure due to varying the column diameter. However, researchers admit to not fully understanding the mechanism contributing to the reduction of the C-term in pcHPLC.[4] Additional positive aspects of pcHPLC include compatibility with flow-limited detectors such as electrospray ionization mass spectrometry (ESI-MS)[5] as well as the increased mechanical strength of the column walls when compared to larger diameter columns. Since the wall strength decreases geometrically as diameter increases, in order to increase the burst-pressure rating of the column wall, thickness must increase rapidly with I.D.[6] Therefore, smaller columns can withstand significantly greater pressures than larger ones. A final advantage to capillary HPLC results from the increased thermal dissipation at lower column diameters.[7] Heat is generated by the resistance of the column to the mobile phase flow and this heat must be dissipated rapidly in order to avoid losses in chromatographic efficiency due to radial heating gradients.[8-10] Radial gradients are easily avoided in capillary columns because the

decreased column diameter results in an increased surface area to volume ratio and a decreased total thermal mass allowing for heat to be more easily dissipated when compared to a large diameter column.

One further development stemming from the development of capillary columns was the ability to use very small (i.e. <2 μm) stationary phase particles which were previously impossible to employ with analytical-scale columns.

1.1.2 Ultrahigh pressure liquid chromatography

Throughout the history of HPLC, smaller diameter stationary phase particles have been sought since their use significantly reduces the van Deemter A-term and C-term contributions which results in greatly enhanced separation efficiency. There has, however, been a limitation to the minimum particle size employed. Since the back pressure afforded by the column increases to the third power as particle diameter decreases[11], column housing materials have limited 4.6 mm columns to only 100 MPa,[12] far lower than the pressures required for the most efficient particle diameters (<2 μm). In addition to the mechanical constraints, the heat generated from forcing the mobile phase through a sub-2 micron particle bed is significant enough to severely reduce the separation efficiency of large diameter columns through the formation of radial thermal gradients.[10] The advances in pcHPLC addressed the problem due to the increased column wall strength and improved thermal dissipation which allowed for the development of ultrahigh pressure liquid chromatography (UHPLC) in the late 1990s.[11]

UHPLC employs specialized equipment to pump mobile phase at pressures as high as 300 MPa through sub-2 micron particle beds, which achieves separation efficiencies an order of magnitude greater than what is possible in conventional HPLC.[13]

In addition, the reduction of C-term contribution to the van Deemter equation due to the smaller particle size allows for faster separations in UHPLC when compared to conventional HPLC with no sacrifice in performance.[14] This increase in separation speed results in vastly improved throughput times with a corresponding reduction in solvent consumption.[15]

Overall, the shrinking of the column I.D. in reduced-scale liquid chromatography successfully reduces the solvent consumption in HPLC with improvements in separation efficiency. There are, however, significant limitations to employing capillary columns in liquid chromatography, mostly stemming from the small volume of the injected sample. In this regard, since the volume of column available for the sample plug to occupy decreases with column I.D., the sample capacity decreases as well.[12] As seen in Table 1-1, a reduction in column I.D. results in an exponential decrease in the maximum amount of sample injected due to the reduction of stationary phase present. This limitation can be challenging for conventional HPLC detection methods such as ultraviolet-visible spectroscopy (UV-Vis) since the low injected mass can form analyte concentrations at the detector that can easily fall below the detector's limit of detection.[16] An additional disadvantage of reduced-scale liquid chromatography arises due to extra-column broadening effects, caused by dead volume in fluidic connections, which become a significant concern when the injected sample occupies such a small volume.[17] In this regard, even the smallest dead volume contribution by a frit or fitting can cause significant reduction in chromatographic efficiency.[18]

1.2 High Temperature Liquid Chromatography

Another method to reduce the solvent consumption in HPLC is to reduce the entire separation time. The limitation to increasing the speed of separation is governed by the van Deemter equation C-term. This resistance to mass transfer increases rapidly with larger mobile phase flow velocities resulting in significant decreases in chromatographic efficiency. One such method of reducing the C-term contribution and therefore allowing for increased separation speed employs heating of the column and mobile phase. High temperature liquid chromatography (HTLC) achieves faster flow rates by decreasing the C-term contribution by increasing the temperature of the mobile phase. The kinetics behind mass transfer are sped up as a result of the decrease in the viscosity and an increase in the analyte diffusivity of the mobile phase.[19] This observation interestingly implies that temperature should have no effect on chromatographic efficiency.[20] Indeed, as temperature increases, the maximum efficiency of a column remains unchanged yet the minima of the van Deemter curve shifts to a higher linear velocity.[21] This trend results in a significant reduction in analysis time without sacrificing chromatographic performance. As such, a shorter analysis time results in a decrease in the amount of mobile phase consumed.

HTLC and reduced-scale liquid chromatography are both successful methods to reduce the consumption of conventional mobile phase solvents but they do not eliminate organic solvent use outright. In order to do so, we must completely replace these eluents with safe, inexpensive and environmentally friendly substitutes. In recent years, some alternate solvent systems have been introduced which do not require any organic solvents in the mobile phase.

1.3 Subcritical Water Chromatography

One such alternate solvent system stems from the development of HTLC but employs pure water as the mobile phase since, among other things, it is inexpensive, safe and environmentally friendly. With the exception of a little-known publication in the early 1980s[22], subcritical water chromatography (SWC) stemmed from the development of subcritical water extraction in the mid 1990s.[23-26]

1.3.1 SWC description

The subcritical moniker arises when water is heated above its boiling point and pressurized enough to maintain it in the liquid phase. The region of the water phase diagram (Figure 1-1) defined as subcritical is above the boiling line and between the room temperature boiling point (100 °C) and the critical temperature (374 °C). Although the definition of subcritical may seem concrete, it is not. Since there is no abrupt change in physical properties when temperature is increased from ambient to above the critical temperature, any of these temperatures may be utilised successfully in a chromatographic or extraction system. However, the upper end of the temperature spectrum may prove troublesome since supercritical water can provide an extremely oxidizing environment and readily corrodes metal alloys and destroys organic molecules.[27] Although, when kept below its critical temperature it is still highly effective. A most interesting and advantageous property of subcritical water is that its polarity significantly lowers as temperature is increased.[28] As shown in Figure 1-2, subcritical water mimics the polarity

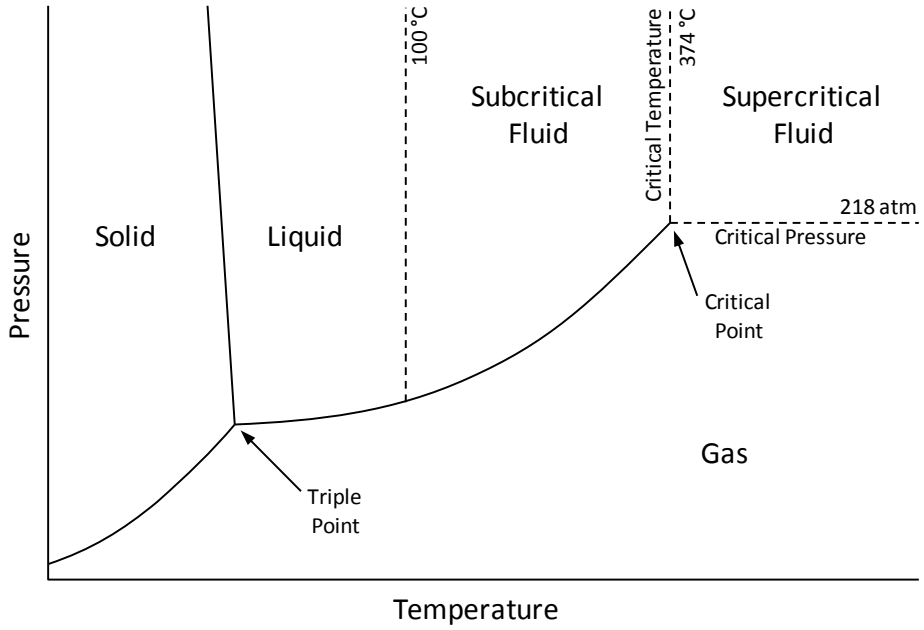


Figure 1-1: Phase diagram of water.

of pure, room temperature acetonitrile at about 160 °C and that of pure, room temperature methanol at about 200 °C.[29] Therefore, a primary advantage of SWC as an alternate separation method is the potential of eliminating conventionally toxic, expensive, and environmentally hazardous organic solvent mobile phases with simple, relatively inexpensive, pure water utilized at select temperatures. Benefits of this approach include the relatively low cost, high purity and environmental compatibility of water relative to typical organic solvents. In addition, akin to HTLC, since SWC is often operated at elevated temperatures, van Deemter C-term contributions are reduced which allows for relatively faster separations.

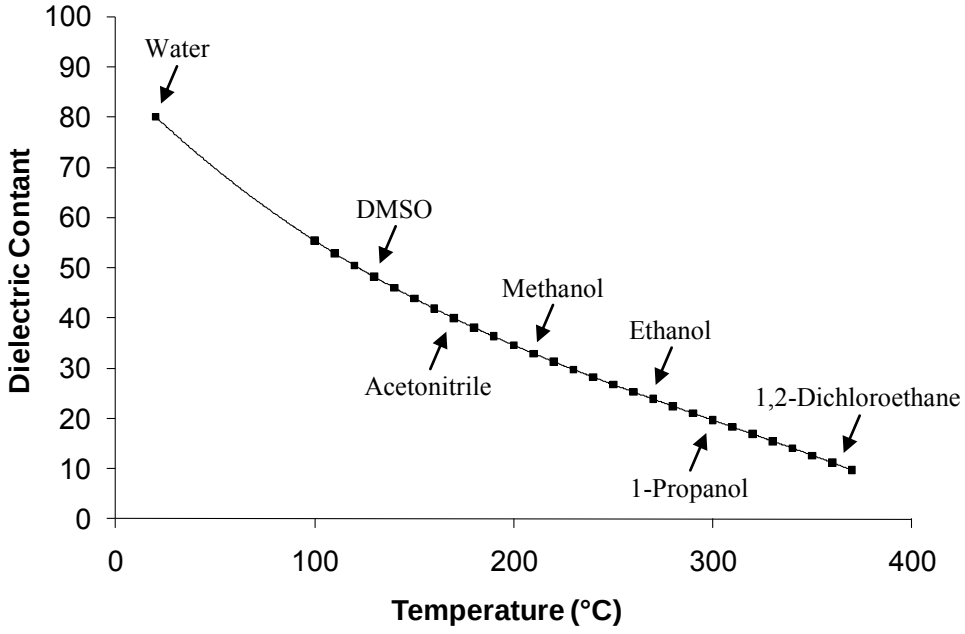


Figure 1-2: Relationship between temperature and the dielectric constant of water. The equivalent room temperature polarities of common HPLC solvents are noted. (Figure compiled from references 28 and 29)

1.3.2 Detection in SWC

Subsequent to the initial few reports on the topic appearing earlier over the last two decades or so,[22,30-31] SWC has evolved into a relatively widely used chromatographic technique.[32-35] The success of SWC can partly be attributed to its compatibility with various forms of HPLC detection including UV-Vis[30,36-55], fluorescence spectroscopy[56], infra-red spectroscopy[50], diode array spectroscopy[57], inductively coupled plasma atomic emission spectroscopy[58], inductively coupled plasma mass spectrometry[59], ESI-MS[41,50,60-61], evaporative light scattering detection[58], refractive index detection[62], conductivity detection[62], corona-charged aerosol detection[63], and proton nuclear magnetic

resonance.[50,64] The most commonly used form of these HPLC detection methods in SWC is UV-Vis. UV-Vis detection is extremely popular in SWC since it is readily adapted from its role as a HPLC detector. Water makes an ideal mobile phase for UV-Vis detection since it has a very low ultraviolet cut-off wavelength of 191 nm.[29] However, UV-Vis detection has its challenges when implemented in SWC. One such limitation stems from the temperature compatibility of the detector cell. Since the UV-Vis detector was designed to operate with ambient temperature mobile phases, a cooling bath is often required to reduce the temperature of the water phase in order to avoid damage to the detector.[33] Tubing coils within this heat exchanger can reduce chromatographic efficiency since they contribute to extra-column broadening. A second limitation to UV-Vis detection results from the specific nature of the detector.[34] In order for UV-Vis to detect an analyte, the compound of interest must contain a chromophore. Since many target analytes do not contain such a chromophore, the application of UV-Vis detection in SWC is therefore limited.

One of the most important benefits of SWC is the compatibility with the desirable universal flame ionization detector (FID).[22,26,31-36,40,42,62,65-77] The FID is classically precluded from use in HPLC since the presence of organic components in the mobile phase blind the detector from responding to analytes. The amenability of SWC to this rugged detection method is a significant advantage for SWC since it offers inexpensive and universal detection.

1.3.3 Heating methods in SWC

Since the polarity of water decreases rapidly with increasing temperature, this feature allows SWC to provide an isocratic mobile phase with thermally tuneable

polarity. As a chromatographic mobile phase in SWC, this property of water is readily manipulated through controlling the temperature of the separation column. Therefore, temperature programming in SWC can be analogous to gradient elution in reversed phase HPLC.[31-36,38-41,45,48,52,55,60,65-68,70-72,77-79] A factor that directly impacts the steepness of the gradient employed in SWC, and hence analysis time, is the rate at which the column temperature can be raised. To date, this parameter has predominantly been controlled using GC convection ovens[30-36,38-41,48,55,60,65-68,70-71,77-78], which typically provide maximum instrumental heating rate settings of 30 to 50 ºC/min.[80] However, an alternative method employing a machined aluminum column heater equipped with a coolant flow capability has also been introduced.[52] This method employs resistive heating with closed-loop temperature control which allows for reproducible column heating to 225 °C. In addition, provisions have been made for pre-heating of the mobile phase to minimise efficiency losses. However, this system is restricted by the relatively large thermal mass of the heater/column assembly which limits the demonstrated heating rate to only 4 °C/min. In this regard, the large thermal mass requires silicone oil for cooling to initial condition. Even with the external cooling fluid, the cooling time is slow and was demonstrated at only -9 °C/min.

Previously, while performing gradient separations in SWC, our research group has observed analyte retention times to converge at increasingly higher temperature programming rate settings when using a conventional GC oven for column heating. For example, Figure 1-3 demonstrates this convergence by displaying two alcohol separations that are nearly identical despite the fact that the temperature programming rate used is

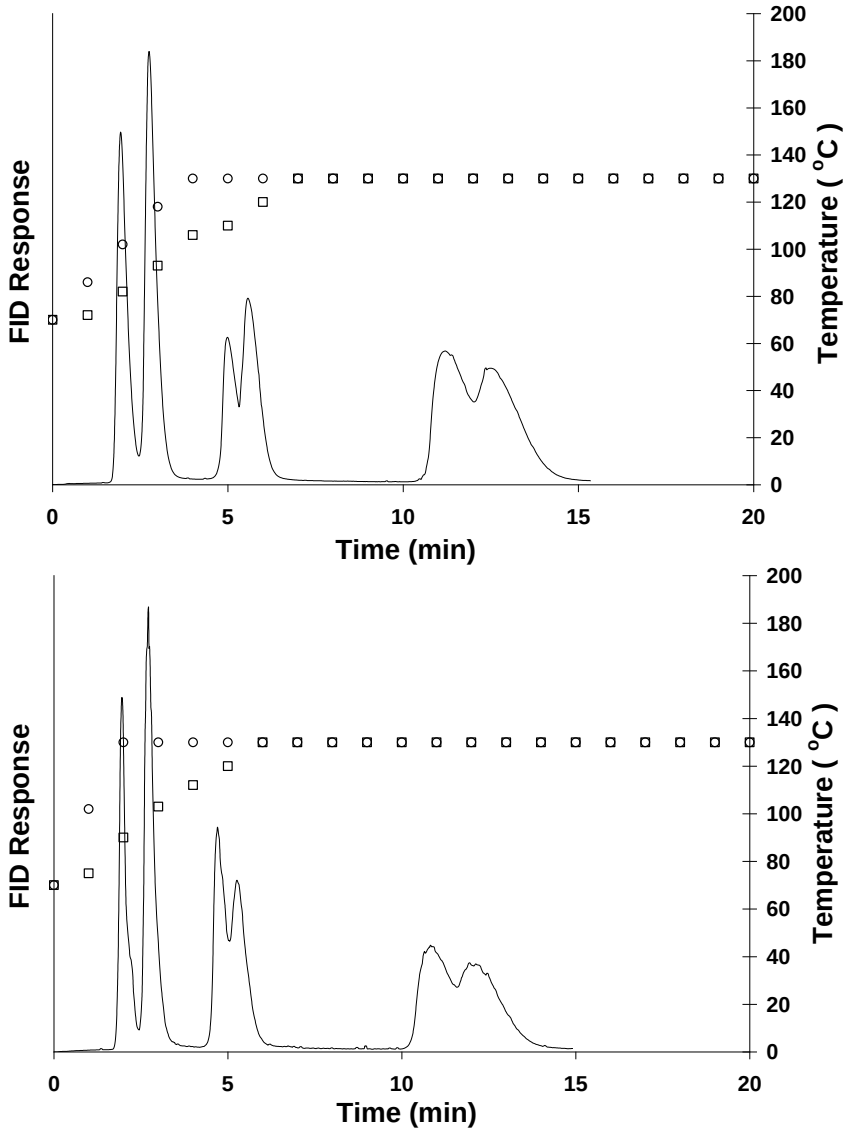


Figure 1-3: SWC separation of alcohols using GC oven heating at programmed rates of 16 (upper) and 32 °C/min (lower). The temperature program for each was 70 °C initial until injection, then increasing at the posted rate to 130 °C final. Overlaid are the observed oven temperature (○) and the actual column temperature (□). The elution order is methanol, ethanol, 2-propanol, 1-propanol, 2-butanol, and 1-butanol. 200 µL/min water, 150 mm × 2.1 mm I.D. (5 µm) PRP-1 column.

doubled from 16 °C/min to 32 °C/min. The inability of the system to further increase analyte peak velocities on the column suggested that the actual mobile phase heating rates had also converged at larger settings. As such, the data in Figure 1-3 reflect that the actual column heating rates achieved for the 16 and 32 °C/min GC oven set value trials are closer to 9 and 10 °C/min, respectively.

Thus, a more effective column heating method that could provide better control over mobile phase temperature would, in turn, be useful in providing better control of analyte retention in SWC analyses. To overcome this obstacle, a novel means of controlling column temperature in SWC has been presented,[72] which is based on resistive heating in GC.[80-83] This alternate heating method uses a resistively heated wire, wound in close contact with the SWC column, to reproducibly raise the column temperature significantly more rapidly than by regular means. Figure 1-4 demonstrates this increase in heating rate with two alcohol separations showing a significant reduction in elution time for the resistively heated separation as compared to the GC oven heating rate. The resistively heated system was able to achieve heating rates of approximately 60 °C/min whereas the GC oven was limited to only approximately 10 °C/min. Therefore, this resistive heating method provides significant improvements over conventional GC convection oven heating systems. The reduction in elution times by more than 50% suggests that resistive heating is a beneficial alternative for use in temperature programmed SWC separations.

Overall, SWC provides a successful technique for replacing organic solvents with inexpensive, safe, and environmentally compatible water. In addition, the amenability to

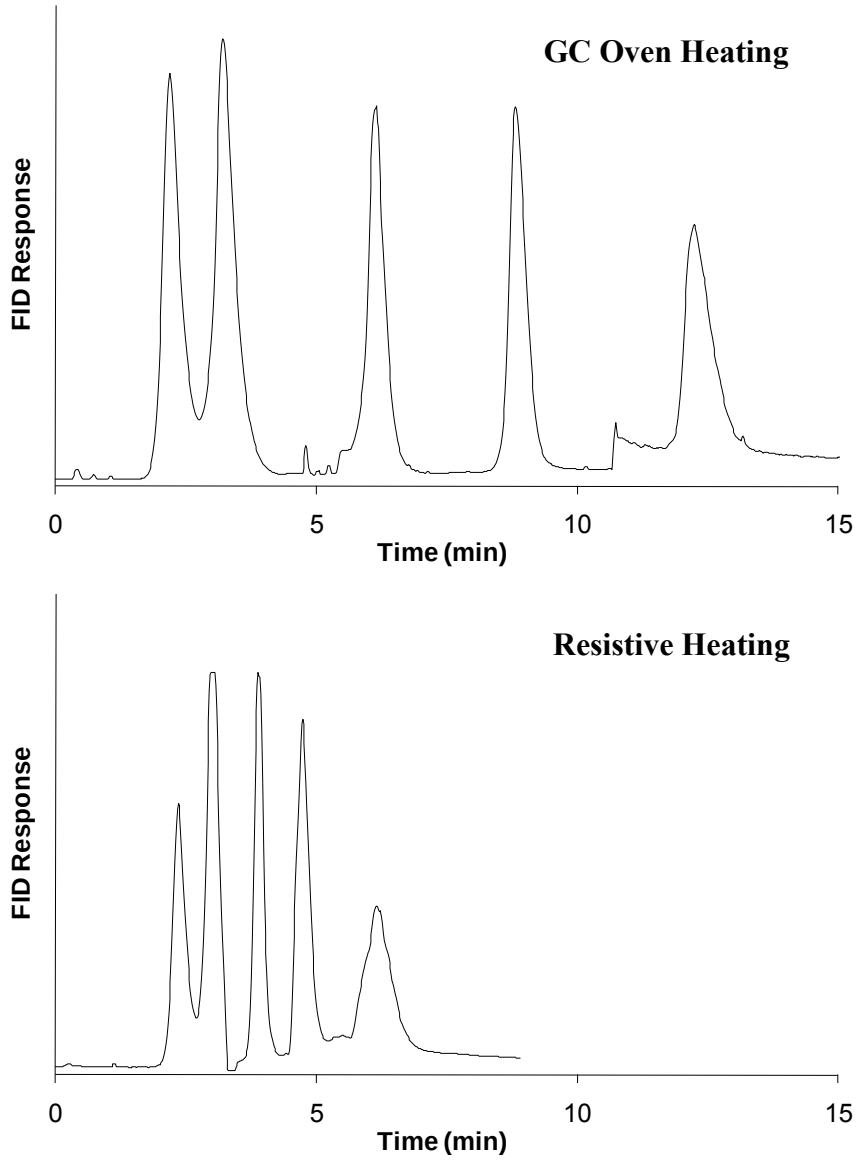


Figure 1-4: SWC separation of an *n*-alcohol mixture using aggressive heating to the largest final temperature attainable. Both the GC oven heating (upper) and resistive heating (lower) modes used their fastest possible heating rates in a program that started at 70 °C for 2 min and then ascended to 200 °C final. The elution order is methanol, ethanol, propanol, butanol, and pentanol. 200 µL/min water, 150 mm × 2.1 mm I.D. (5 µm) PRP-1 column.

the FID, which was previously not available in HPLC, makes SWC an excellent alternative solvent system for reducing the consumption or organic solvents in liquid chromatography.

1.4 Supercritical Fluid Chromatography

Supercritical fluid chromatography (SFC) has evolved over the past 50 years or so[84] into an alternate solvent system which performs separations in a similar fashion to HPLC.[85] As shown in Figure 1-5, a supercritical fluid resides on the phase diagram above the critical point whereby any further increase in pressure will not result in condensation and any further increase in temperature will not result in evaporation. This phase has properties in between that of a liquid and a gas. For example, a supercritical fluid has liquid-like solvating power yet retains a gas-like diffusivity. These intermediate properties of a supercritical fluid make it an interesting alternative to traditional chromatographic mobile phases. For example, the increased diffusivity of a supercritical fluid relative to a liquid translates to reduced C-term contribution to the van Deemter equation. In addition, a supercritical fluid has considerably reduced viscosity over a liquid which allows for the use of longer columns and/or smaller diameter stationary phase particles in this mode of chromatography. The combination of a reduced C-term and the availability of higher efficiency columns result in SFC achieving greater efficiency and faster separations as compared to HPLC.[86] In addition to the efficiency advantages, SFC also allows for unique control over selectivity. Since temperature and pressure alter the density (solvating power) of a supercritical fluid and temperature

changes the vapour pressure (solvation) of the analyte, both of these parameters are available to optimize SFC separations.

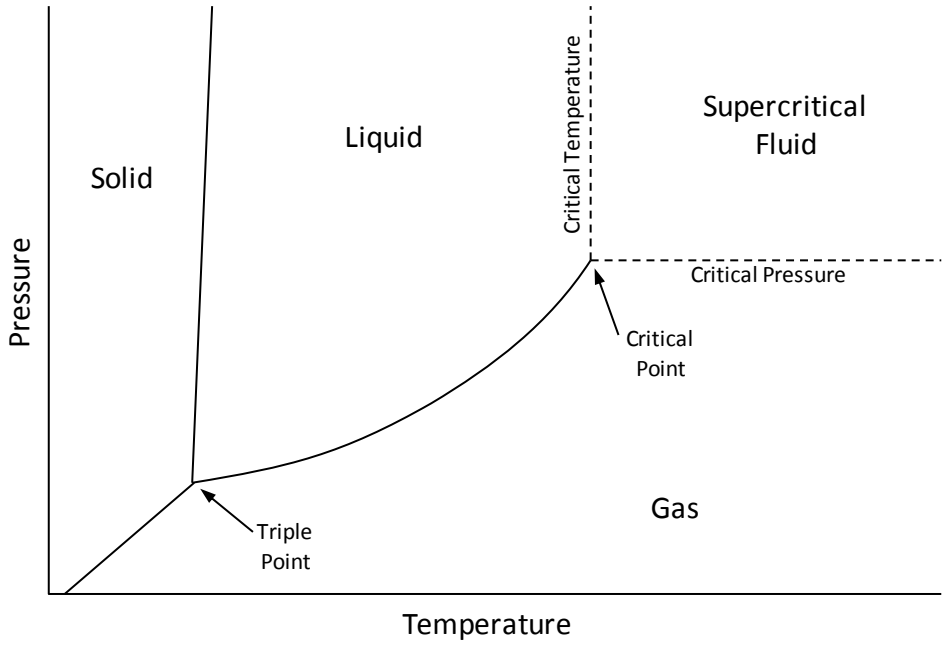


Figure 1-5: Phase diagram for SFC.

Fluids that have been employed as SWC mobile phases include ammonia[87], sulphur dioxide[88], nitrous oxide[89], hydrocarbons[90], chlorofluorocarbons[91], fluorocarbons[92], sulphur hexafluoride[93], xenon[94], and water.[95] But, since most of these options are unsafe, corrosive and/or expensive they have not garnered much interest in the field. However, CO_2 has become the fluid of choice for SFC due to its modest critical parameters (31.03 °C, 72.83 atm)[96], low cost, available purity, safety and ease of use.[97] Also contributing to its popularity, CO_2 has a ultraviolet cut-off wavelength below 200 nm[98] and it does not respond in the FID.[95] In addition and if desired, sample collection is

made easy since, when depressurized, the CO_2 mobile phase evaporates which leaves only the concentrated analyte in a relatively small volume of solvent. As such, CO_2 SFC presents an alternative separation system which does not consume traditional HPLC organic solvents.

These advantages have garnered a considerable industrial following for SFC largely owing to its amenability to high-purity, large-scale separations (i.e. kg per day)[99] and to its amenability to separate chiral mixtures.[100] Due to these advantages, the pharmaceutical industry has particularly embraced SFC since it often requires very high purity, preparative-scale separations of chiral compounds. In addition, on a small preparative scale, it is uncommon to re-use solvents in industrial settings. Therefore, since CO_2 is inexpensive to procure and dispose of, SFC can reduce the operating costs relating to chromatographic separations. An additional advantage of SFC which appeals to industry is the ‚green' aspect of SFC. Since CO_2 is an environmentally compatible solvent, and often an alternative to petroleum-derived solvents such as hexane or heptane, marketing departments can exploit this attribute to attract the market share of environmentally-conscious consumers. Further, CO_2 can and is frequently re-used if desired.

However, a major limitation of CO_2 SFC results from the non-polar nature of CO_2 which is akin to that of pentane.[97] Therefore, the solvent strength of CO_2 may not be able to successfully elute all but the most non-polar analytes. In order to improve the solvent strength toward polar analytes, polar co-solvents such as methanol are often blended with pure CO_2. While the addition of these modifiers can expand the amenability of CO_2 SFC

toward more polar solutes, it counteracts the ability of SFC to replace organic solvents and precludes the use of the FID due to the presence of carbon in the mobile phase.

1.5 Motivations for This Research

As presented above, subcritical water becomes much less polar as temperature is increased. However, this process spans a considerable range over several hundred degrees Celsius.[26] Unfortunately, the lack of thermal stability of column stationary phases and analytes over this same range limits the minimum water polarity attainable and therefore also the elution strength of the mobile phase.[101-102] For example, well cross-linked bonded-phases on silica have a thermal ceiling of about 150 °C while polymeric phases often begin to degrade around 200 °C.[103] However, even at 200 °C, the polarity of water mimics just that of pure, room temperature methanol.[26,28-29] Conversely, a non-polarity akin to dichloroethane requires temperatures approaching 425 °C.[29,104] As such, SWC is conventionally often ineffective at eluting and analyzing non-polar analytes.

While efforts have been made to advance SWC areas such as column heating methods[45,52,72] and stationary phase thermal stability[44,105-106], the conventional water mobile phase has remained unaltered for the most part.[32-35,65] In this regard, the addition of a non-polar modifier to the mobile phase has the potential of increasing the solvent strength of the mobile phase without the need for excessively high temperatures. One such non-polar modifier that has not been explored in SWC is CO_2. CO_2 makes an ideal modifier for SWC since it shares many of the same ‚green' aspects of water such as redeuced toxicity and environmental compatibility. In addition, since CO_2 does not

respond in the FID, the addition of CO_2 to SWC preserves the ability to use the FID in this mode of chromatography.

This thesis will describe in depth, novel investigations into using CO_2 and water together in various separation schemes. Chapter Three describes blending of non-polar CO_2 with subcritical water as a mobile phase in SWC in order to increase the solvating power of the mobile phase while reducing the operating temperature. Chapter Four then describes the application of the CO_2 modified SWC system to elute a model pharmaceutical in order to explore the possibility of adapting this novel separation technique to pharmaceutically relevant separations. In Chapter Five, attempts are made to investigate the possibility of applying ultrasound to the CO_2 modified SWC system in order to promote better mixing, which would result in better chromatographic performance at lower temperatures which, in turn, could further expand the application of this mode of chromatography. Based on a unique observation, Chapter Six details a novel separation system as performed in a capillary tube using water (saturated with CO_2) as a stationary phase and CO_2 (saturated with water) as a mobile phase. Chapter Seven next presents the results of exploring this system by loading the water stationary phase with various water-soluble components in efforts to change the retention of select analytes. Finally, Chapter Eight is comprised of a summary and thoughts on future work in this area. Each chapter also presents additional introductory remarks with a particular focus on the topical area being covered.

Chapter Two: **EXPERIMENTAL**

2.1 Carbon Dioxide Modified Subcritical Water Chromatography

2.1.1 Instrumentation

Figure 2-1 shows a schematic diagram of the modified SWC-FID system used in Chapter Three which is similar to one used previously in our laboratory.[72] The convection oven and FID system were provided by a Shimadzu model GC-8A gas chromatograph (GC) (Shimadzu, Kyoto, Japan). The blended mobile phase was delivered using two Isco model 260D syringe pumps (Teledyne Isco Inc., Lincoln, NE, USA). The water pump was operated in constant flow mode at a set rate of 200 μL/min. Liquid CO_2 was drawn into a second pump which was operated in constant pressure mode and the CO_2 flow rate varied accordingly with the set pressure. The latter pump was also equipped with a water cooling jacket operated at about 10 °C to ensure greater filling efficiency and reliable fluid metering.

Each pump outlet was connected to an individual 2.8 m length of coiled stainless steel tubing (1/16 in. O.D. × 0.010 in. I.D.; Chromatographic Specialties Inc., Brockville, ON, Canada) which led through the oven wall and pre-heated the mobile phase components. Pre-heating the mobile phase is important in order to reduce the presence of thermal gradients in the column which will result in efficiency loss.[12] The respective pump lines were then joined inside the oven in a 1/16 in. Valco zero dead volume (ZDV) stainless steel tee union (Alltech Associates, Inc., Deerfield, IL, USA). A coiled 1 m length of the same tubing led from the tee and allowed further pre-heating of the mobile phase mixture before briefly exiting the GC oven and connecting to a Rheodyne model

7520 injector (Alltech) equipped with a 0.5 µL sample loop. A 15 cm length of the same tubing then led from the injector, back through the GC oven wall, to the analytical column.

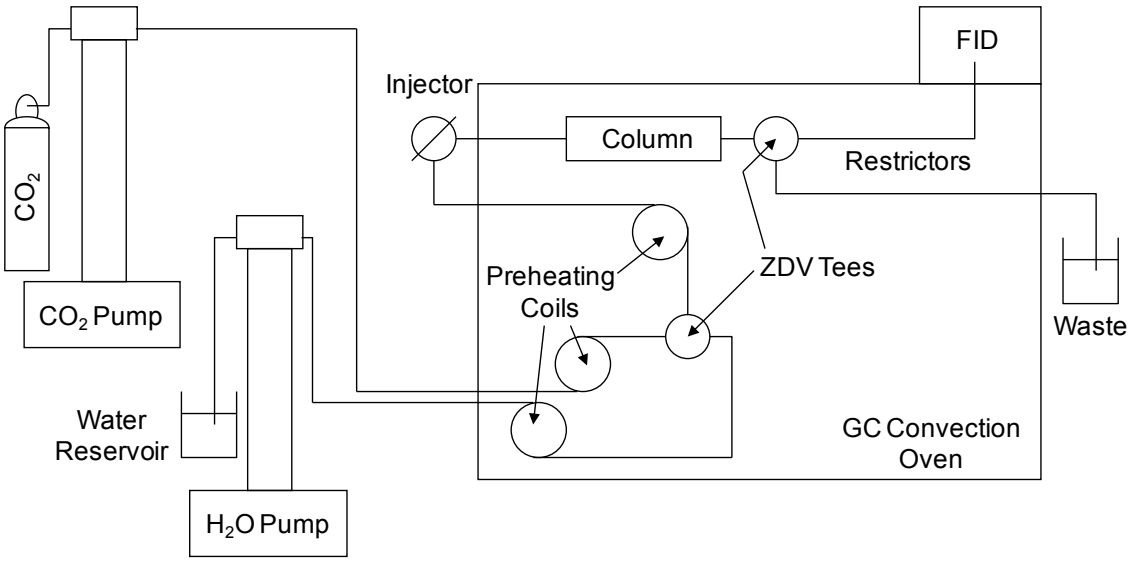


Figure 2-1: Schematic diagram of the CO_2 modified SWC system.

Separations were performed on a 50 mm × 4.1 mm I.D. Hamilton PRP-1 column containing a poly(styrene-divinylbenzene) stationary phase (5 µm spherical particles; Hamilton Company, Reno, NV, USA). A 2 cm length of the same tubing led from the column outlet to a second Valco ZDV stainless steel tee union (Alltech). One outlet of the union was routed to waste via a 30 cm length of fused silica tubing (50 µm I.D.; Polymicro Technologies, Phoenix, AZ, USA). The second outlet of the tee was connected to the FID via a length of the same fused silica tubing, which was normally kept between 30 and 45 cm in order to maintain flame stability and system back pressure. At a

temperature of 150 °C with a water flow rate of 200 μL/min and a CO_2 flow rate of 636 μL/min this restrictor assembly typically achieved a system back pressure of 200 atm. The outlet of this transfer line was placed at the base of the FID jet within the detector housing, where it provided the best performance in terms of flame stability and response.

The FID gas flow rates were typically about 350 mL/min of air and 100 mL/min of hydrogen. However, similar to other SWC reports[31], the latter was occasionally increased as needed to maintain flame stability at different column flow rates. The detector block was held at 350 °C.

2.1.2 Column cleaning procedure

To ensure reproducibility and longevity of the PRP-1 separation column, a cleaning procedure was frequently employed. This procedure involved back-flushing 160 atm of 120 °C CO_2 through the column for one hour followed by a 2 °C/min negative temperature program to 20 °C where it was held for a further 30 minutes. Occasionally, when further cleaning was warranted, the CO_2 cleaning procedure was preceded by a reverse-flow rinse of 15 mL of each of water, ethanol, hexanes, and finally isopropanol. This solvent rinse was consistently followed by the CO_2 cleaning procedure in order to help ensure that any residual organic components were flushed from the column prior to performing separations.

2.1.3 Chemicals and reagents

Instrument-grade CO_2 (99.99%; Praxair, Calgary, AB, Canada) and nitrogen-purged HPLC-grade water (2 MΩ cm; Thermo Fischer Scientific, Hampton, NH, USA) composed the mobile phase. Analyte solutions were prepared by dissolving standards (all ≥99%; Sigma-Aldrich, Oakville, ON, Canada) in HPLC-grade solvents (Sigma-Aldrich).

The specifics of these standards such as composition and concentration are shown in Table 2-1. All other details and variations are described in Chapter Three.

Table 2-1: Composition and concentration of analyte standards employed in Chapter Three.

Solution	Analyte	Concentration (mg/mL)	Solvent
Butanol test analyte	1-Butanol	63.20	Water
n-Alcohol mixture	Methanol	106.49	Water
	Ethanol	99.30	
	1-Propanol	50.70	
	1-Butanol	53.40	
	1-Pentanol	51.04	
	1-Hexanol	52.82	
	1-Heptanol	48.22	
	1-Octanol	50.12	
Acetophenone derivatives	3'-Hydroxyacetophenone	103.45	Acetone
	Acetophenone	109.39	
	3'-Methylacetophenone	213.54	
Aniline derivatives	Aniline	10.83	Dichloromethane
	3-Chloroaniline	10.24	
	2,3-Dichloroaniline	10.54	
	2,4,5-Trichloroaniline	11.26	
BTEX mixture	Benzene	100.98	Methanol
	Toluene	100.12	
	Ethylbenzene	102.19	
	para-Xylene	99.62	
Short chain carboxylic acids	1-Butanoic acid	99.75	Isopropanol
	1-Hexanoic acid	101.22	
	1-Octanoic acid	100.68	
	1-Decanoic acid	101.28	
Free fatty acids	Myristic acid (14:0)	19.63	Isopropanol
	Palmitic acid (16:0)	19.03	
	Stearic acid (18:0)	19.16	

Brij-52® solution	Polyoxyethylene (n~2) cetyl ether	104.61	Methanol
PEG 200 solution	Polyethylene glycol (Ave MW 200 g/mol)	104.90	Water
PEG 400 solution	Polyethylene glycol (Ave MW 400 g/mol)	104.59	Water
PEG 600 solution	Polyethylene glycol (Ave MW 600 g/mol)	103.78	Water
PEG 1000 solution	Polyethylene glycol (Ave MW 1000 g/mol)	101.60	Water
Peptide mixture	Met	~1.0*	Methanol
	Gly-Tyr	~0.5*	
	Val-Tyr-Val	~1.0*	
	Tyr-Gly-Gly-Phe-Met	~1.0*	
	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	~1.0*	
Pressure-programmed carboxylic acids	1-Hexanoic acid	50.50	Isopropanol
	1-Decanoic acid	50.30	
	1-Tetradecanoic acid	53.85	
	1-Octadecanoic acid	63.63	
Column recovery test mixture	Methanol	101.42	Water
	Ethanol	102.92	

* A solid residue of the peptide mixture was dissolved in a minimum volume of methanol, forming the estimated concentrations listed.

2.2 Exploring a Model Pharmaceutical with SWC and CO_2 Modified SWC

2.2.1 Instrumentation

The CO_2 modified SWC system used in Chapter Four is identical to that used in Chapter Three, as described in section 2.1.1 above. Conventional SWC analysis was performed with the same apparatus but with the CO_2 pump closed off to the system. Multiple columns were explored in this mode including a PRP-1 column, a polybutadiene(PBD)-clad zirconia column, a cyano-bonded silica column, and a bare silica packed capillary column. The PRP-1 column (150 mm × 2.1 mm I.D.; 5 μm spherical particles; Hamilton) was operated with a 150 μL/min water flow rate and

system restriction was provided by a 40 cm length of 50 μm I.D. fused silica tubing (Polymicro). The PBD-clad zirconia column (100 mm × 2.1 mm I.D.; 3 μm spherical particles; ZirChrom Separations, Inc., Anoka, MN, USA) was operated with a 150 μL/min water flow rate and system restriction was provided by a 40 cm length of 50 μm I.D. fused silica tubing (Polymicro). The cyano-bonded silica column (250 mm × 4.6 mm I.D.; 5 μm spherical particles; Alltech) was operated with a 500 μL/min water flow rate. System restriction was provided by two lengths of 50 μm I.D. fused silica tubing (Polymicro) arranged to split the column flow with a 21 cm length directed to waste and a 45 cm length leading to the detector. This arrangement provided approximately a 3:1 split ratio. The bare silica packed capillary column (50 mm × 250 μm I.D.; 5 μm irregular particles; prepared in-house) was operated with a 10 μL/min water flow rate and system restriction was provided by a 30 cm length of 50 μm I.D. fused silica tubing (Polymicro). To help ensure that no fluoxetine hydrochloride ($C_{17}H_{19}F_3NOCl$) remained on these columns after SWC elution, each column was flushed with acetonitrile followed by water prior to each subsequent analysis.

HPLC elution was performed in a similar fashion with an acetonitrile mobile phase pumped at 150 μL/min. Detection was provided by a Waters model 440 absorbance detector (254 nm; Waters Corporation, Milford, MA, USA). A split was not employed for HPLC analysis.

2.2.2 Column packing procedure

The column prepared in-house was packed in fused silica tubing (Polymicro) using Valco ZDV stainless steel unions (Alltech) with 1/16 in. stainless steel frits (0.5 μm pores; Chromatographic Specialties Inc.) as end fittings. Tubing adapters were employed

to minimize dead volume. The column was packed with 5 μm irregular silica particles (Silicycle Inc., Quebec City, QC, Canada) using a CO_2/methanol slurry packing method that was derived from previously published techniques.[107] The packing procedure involved preparing a slurry of stationary phase particles in methanol which was forced through the column with CO_2. The CO_2 pressure began at 100 atm and was gradually increased to 220 atm where it was held for about 30 minutes to ensure complete packing. The packed column was then allowed to slowly depressurize to ambient pressure before the inlet fitting was installed. The column tube was immersed in a VWR 50D ultrasonic cleaning bath (1.9 L, 35 kHz, 45 W; VWR International, Edmonton, AB, Canada) throughout the packing procedure with the ultrasound activated in order to aid in the packing process.

2.2.3 Chemicals and reagents

Instrument-grade CO_2 (99.99%; Praxair), nitrogen-purged HPLC-grade water (2 MΩ cm; Fischer), and HPLC grade acetonitrile (Sigma-Aldrich) composed the mobile phases employed. The fluoxetine solution was prepared to 10.78 mg/mL in HPLC-grade water (2 MΩ cm; Fischer). The fluoxetine standard was a generous gift from Eli Lilly and Company, Indianapolis, IN, USA. All other details and variations are described in Chapter Four.

2.3 Ultrasound in CO_2 modified SWC

2.3.1 Instrumentation

The system used in Chapter Five is similar to the CO_2 modified SWC system described in section 2.1.1 above. However, in place of the GC convection oven, the VWR

50D ultrasonic cleaning bath was used to provide heat for the system as well as a source of ultrasound as seen in Figure 2-2. Additional ultrasonic energy was provided by a Cole Palmer CP134PB ultrasonic processor (40 kHz, 130 W ; Cole Palmer Canada Inc., Montreal, QC, Canada) with a custom-built tip which allowed for direct contact with various components of the separation system. In this regard, a localized region high in ultrasonic energy could be applied to specific areas of the system such as the mixing tee, the preheating coils, or anywhere along the separation column. A Barnant model 600-1000 J-type digital thermocouple (Barnant Company, Barrington, IL, USA) was used to monitor the temperature of the ultrasonic bath.

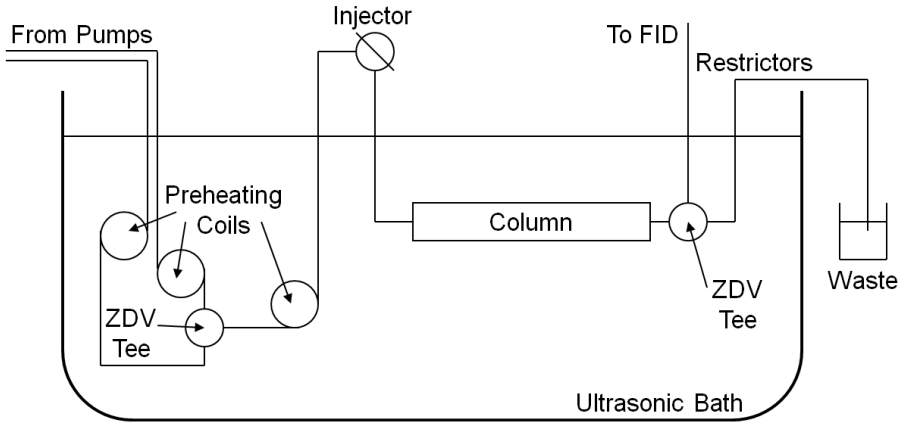


Figure 2-2: Schematic diagram for the application of ultrasound to the CO_2 modified SWC system.

Conventional SWC analysis was performed with the same apparatus but with the CO_2 pump closed off to the system. The pure water mobile phase was pumped at 1 mL/min through the column and the system was maintained at about 70 °C. In this case, the split ratio was increased to about 10:1 to e nsure FID stability. HPLC analysis was

performed in a similar fashion with a methanol/water mobile phase (60:40) pumped at 1 mL/min. The system was maintained at about 30 °C. Detection was provided by a Waters model 440 absorbance detector (254 nm; Waters). No split was employed for HPLC analysis.

SFC experiments were also performed using the same instrumentation with the water pump closed off from the system. For these separations, two separation columns were employed. The first was the PRP-1 column above and the second was a 15 cm × 250 μm I.D. packed capillary column was used that was prepared in-house. System restriction for the PRP-1 column was provided by a 1 m straight length of 50 μm I.D. fused silica tubing (Polymicro). The packed capillary column employed a 20 cm length of 50 μm I.D. fused silica (Polymicro) as a transfer line from the injector to the column and a 40 cm length of the same tubing provided system restriction. PEEK tubing adapters (1/16 in. O.D. × 395 μm I.D.; Idex Health & Science, Oak Harbor, WA, USA) were used in all fittings to reduce dead volume. Separations were performed at 100 atm and approximately 60 °C.

2.3.2 Column packing procedure

The column prepared in-house for use in this chapter was packed in the same fashion as section 2.2.2 above.

2.3.3 Chemicals and reagents

Instrument-grade CO_2 (99.99%; Praxair), nitrogen-purged HPLC-grade water (2 MΩ cm; Fischer), and HPLC grade methanol (Sigma-Aldrich) composed the mobile phases employed. Analyte solutions were prepared by dissolving standards (all ≥99%; Sigma-Aldrich) in HPLC-grade solvents (Sigma-Aldrich). The specifics of these

standards such as composition and concentration are shown in Table 2-2. All other details and variations are described in Chapter Five.

Table 2-2: Composition and concentration of analyte standards employed in Chapter Five.

Solution	Analyte	Concentration (mg/mL)	Solvent
HPLC test mixture	Benzene	34.96	Hexanes
	Toluene	34.60	
SWC test mixture	Methanol	101.42	Water
	Ethanol	102.92	
SFC test analyte	1-Octanol	8.24	CS_2
Mixed mobile phase test analyte	1-Butanol	63.20	Water

2.4 Chromatography Using a Water Stationary Phase and a CO_2 Mobile Phase

2.4.1 Instrumentation

Figure 2-3 provides a diagram of the separation system used in Chapter Six which is an extension of the system used in the CO_2 modified SWC studies.[108] Water and CO_2 were introduced into the system by twin Isco 260D syringe pumps (Teledyne Isco Inc.). The CO_2 pump was operated in constant pressure mode. While the CO_2 flow rate varied accordingly with the set system pressure, it could be independently controlled by varying restrictor length as needed. This pump was equipped with a water cooling jacket operated at about 10 °C to ensure greater filling efficiency and effective fluid metering. When required, the water pump was operated in constant flow mode where the flow rate was optimized as necessary to maintain the water stationary phase at different temperatures.

Each pump outlet was connected to an individual 2.8 m length of coiled stainless steel tubing (1/16 in. O.D. × 0.010 in. I.D.; Chromatographic Specialties Inc.) that led into a Shimadzu model GC-8A gas chromatograph (Shimadzu) convection oven where it pre-heated the water and CO_2. The respective pump lines were joined inside the oven in a 1/16 in. Valco ZDV stainless steel tee union (Alltech). A coiled 1 m length of the same tubing led from the tee and allowed for further pre-heating before briefly exiting the GC oven and connecting to a Rheodyne model 7520 injector (Alltech) equipped with a 0.5 µL sample loop.

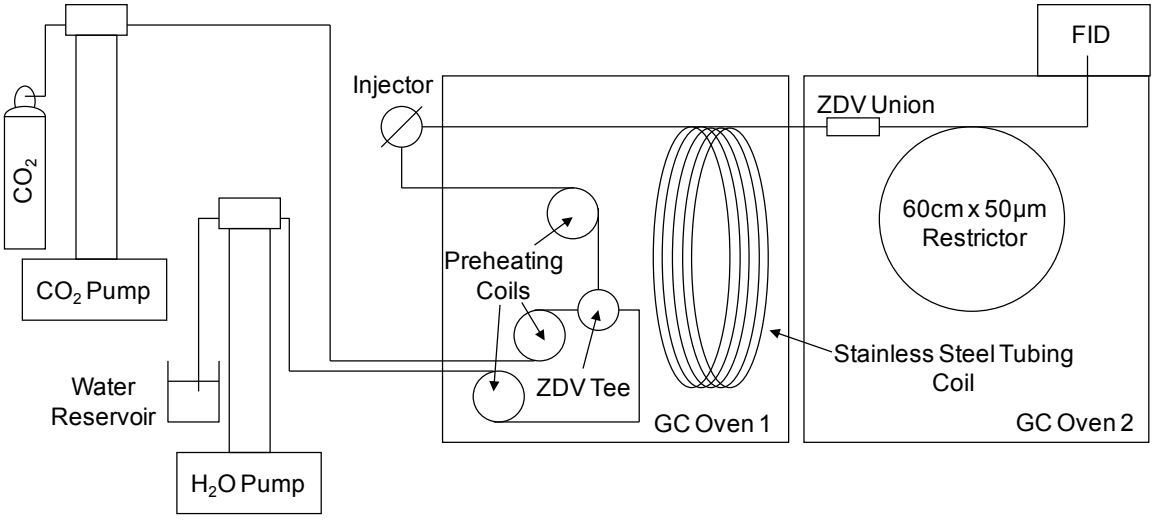


Figure 2-3: Schematic diagram of the separation system using water as a stationary phase and CO_2 as the mobile phase.

The separation column, a 10 m length of the same tubing, was connected to the outlet of the injector, led through the GC oven wall and was coiled inside. All experiments were performed with this 250 µm I.D., 10 m column, except where noted in

the text. A short length of the end of the column exited the separation oven and led into a second Shimadzu model GC-8A gas chromatograph (Shimadzu) convection oven which provided convenient, independent heating of the restrictor and the FID system for certain low temperature separations. However, normally a single oven could also be used for both and still provide good separations although it makes it more difficult to heat the restrictor at lower column temperature settings if required. It should be noted that under these conditions, no corrosion was evident in the 250 μm stainless steel capillary after routine use of the same 10 m length of tubing over several months of study.

System restriction was provided by a 60 cm length of 50 μm I.D. fused silica tubing (Polymicro Technologies) heated to an optimized minimum value of 180 °C. This temperature and the restrictor length were also occasionally altered to provide some control over CO_2 flow rate, similar to previous capillary SFC examples.[109] For example, increasing from 180 °C to 300 °C netted a linear, 15% reduction in flow rate over this range. The end of the restrictor was placed inside the FID jet about 2 cm from the tip where it provided the best performance in terms of flame stability and response. Fused silica restrictor tubing was selected due to its availability in small bore sizes. However, due to silica's erosion in hot water, the restrictors had limited lifetimes. When this erosion became problematic, the system would yield greatly varying flow rates. As such, they were replaced regularly to ensure flow rate reproducibility. Stainless steel is likely a better choice for restrictor material where available. It was found that restrictor temperatures below 180 °C resulted in considerable FID flame flicker noise with occasional flame outages. However, above this temperature, smooth detector operation was observed.

The FID gas flow rates were typically about 350 mL/min of medical-grade air (Praxair) and about 50 mL/min of hydrogen (Praxair). The latter was occasionally increased as needed to maintain flame stability at different column flow rates. The detector block was held at 350 °C.

Conventional GC analyses were performed with a Hewlett-Packard model 5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA, USA) employing split injection (5.4:1) onto an Alltech Econo-Cap EC-5 (5%-phenyl, 95%-methylpolysiloxane) megabore column (20 m × 0.53 mm I.D., 1.5 μm phase thickness; Alltech). High purity helium (Praxair) was used as a carrier gas and operated at about 7 mL/min. A FID was employed with hydrogen (Praxair) and medical-grade air (Praxair) flowing at about 30 mL/min and 300 mL/min respectively. The injector and detector blocks were maintained at 250 °C.

2.4.2 Establishing the water stationary phase

The water stationary phase can be established inside the column by one of two ways. The first involves setting the oven temperature and the CO_2 pump pressure to the desired values and allowing the system to equilibrate to a stable flow rate. Next, the water flow rate is initiated and maintained until water is observed at the column outlet. Finally, the water flow rate is then lowered to the desired level, the FID is ignited, and separations are performed. The second method again involves setting the oven temperature and the CO_2 pump pressure to the desired values; this time, however, with the CO_2 pump closed off from the system. The water pump then completely fills the column until a steady stream of water is observed exiting the restrictor. At this point, the CO_2 pump is opened to the system and the water pump is set to the desired flow rate. Finally, the system is

allowed to equilibrate until the CO_2 flow stabilizes, the FID is then ignited and separations are performed.

2.4.3 Chemicals and reagents

Instrument-grade CO_2 (99.99%; Praxair) and nitrogen-purged, HPLC-grade water (2 MΩ cm; Fischer) were employed as the mobile phase and stationary phase respectively. Analyte standards were prepared by dissolving test compounds (all ≥99%; Sigma-Aldrich) in HPLC-grade solvents (Sigma-Aldrich). The tocopherol samples were a generous gift from the Heyne lab of the Department of Chemistry, University of Calgary. The specifics of these standards such as composition and concentration are shown in Table 2-3. All other details and variations are described in Chapter Six.

Table 2-3: Composition and concentration of analyte standards employed in Chapter Six.

Solution	Analyte	Concentration (mg/mL)	Solvent
n-Alcohol mixture	Methanol	10.70	Water
	Ethanol	10.38	
	1-Propanol	10.39	
	1-Butanol	9.98	
	1-Pentanol	11.10	
Phenol and methanol mixture	Methanol	11.46	Water
	Phenol	10.51	
Menthol test analyte	Menthol	9.97	Methanol
Alkane mixture	n-Hexane	10.41	CS_2
	n-Decane	10.42	
Citric acid solution	Citric acid	20.80	Water
Propanoic acid solution	1-Propanoic acid	10.23	Water

Propanol solution	1-Propanol	10.45	Water
Propanal solution	1-Propanal	10.59	Water
Propanediol solution	1,2-Propanediol	10.89	Water
Butanoic acid solution	1-Butanoic acid	10.50	CS_2
Butanol solution	1-Butanol	9.89	CS_2
Butanethiol solution	1-Butanethiol	10.63	CS_2
Butylamine solution	1-Butylamine	10.96	Water
Chlorobutane solution	1-Chlorobutane	9.90	CS_2
Butyl ethanoate solution	Butyl ethanoate	10.01	CS_2
Butyl ether solution	Butyl ether	10.22	CS_2
Methyl isobutyl ketone solution	Methyl isobutyl ketone	11.27	CS_2
Tertiary alcohol solution	2-Methyl-2-butanol	9.76	CS_2
Tributyl phosphate solution	Tributyl phosphate	10.70	CS_2
Benzene solution	Benzene	10.54	CS_2
Pyridine solution	Pyridine	10.16	CS_2
2 mg Tocopherol solution	Tocopherol	2.23	CS_2
10 mg Tocopherol solution	Tocopherol	11.36	CS_2
Methylated tocopherol	Tocopherol, methyl ether	2.11	CS_2
Octadecanoic acid solution	1-Octadecanoic acid	6.13	10 % 1-butanoic acid in CS_2 (v/v)
Propanol sample capacity A	1-Propanol	11.23	Water
Propanol sample capacity B	1-Propanol	102.30	Water

Propanol sample capacity C	1-Propanol	200.31	Water
Propanol sample capacity D	1-Propanol	N/A – neat injection	N/A – neat injection
Pentanol sample capacity A	1-Pentanol	10.03	CS_2
Pentanol sample capacity B	1-Pentanol	101.23	CS_2
Pentanol sample capacity C	1-Pentanol	200.40	CS_2
Pentanol sample capacity D	1-Pentanol	N/A – neat injection	N/A – neat injection
Carboxylic acid solution	1-Propanoic acid 1-Butanoic acid 1-Pentanoic acid 1-Hexanoic acid 1-Heptanoic acid 1-Octanoic acid	11.13 9.96 11.35 9.67 10.95 10.09	Ethanoic acid
Alcoholic beverage standard	Ethanol 1-Propanol	7.89 8.03	Water
Burbon analysis	Burbon whiskey 1-Propanol	2 % (v/v) 8.03	Water
Wine analysis	Red wine 1-Propanol	2 % (v/v) 8.034	Water
Caffeine standard	Caffeine	9.61	Water
Energy beverage	Energy beverage	N/A – neat injection	N/A – neat injection
Coffee	Tim Horton's coffee	N/A – neat injection	N/A – neat injection
GC ethanol standard	Ethanol	7.89	CS_2
GC gasoline analysis	Ethanol blended gasoline	10 % (v/v)	CS_2
Conventional gasoline	Conventional gasoline	N/A – neat injection	N/A – neat injection
Ethanol blended gasoline	Ethanol blended gasoline	N/A – neat injection	N/A – neat injection
Brij®-30 solution	Polyoxyethylene (n~4) lauryl ether	10.12	Water

2.5 Chromatography Using a Modified Water Stationary Phase

2.5.1 Instrumentation

Chapter Seven employs a similar separation system to the water stationary phase apparatus,[110] however, it uses a 1 m length of 250 μm I.D. stainless steel tubing as the separation column. In addition, the water pump is removed from the system and separations are performed at room temperature to reduce the likelihood of stationary phase evaporation.

2.5.2 Establishing the modified water stationary phase

In order to establish a modified water stationary phase, the column is first removed from the system and filled manually using a syringe with the stationary phase of interest. The column is then re-installed and the CO_2 pump is opened to the system, having already been set at the desired pressure. Finally, the system is allowed to equilibrate until the CO_2 flow stabilizes, the FID is then ignited and separations are performed.

2.5.3 FID cleaning procedure

The presence of ionic compounds in the stationary phase can potentially result in some of these compounds depositing in the detector. Therefore, in order to maintain optimal performance, a detector cleaning procedure was frequently employed. Since all of the stationary phase additives are water-soluble, the cleaning procedure simply involved fully disassembling the detector and rinsing all of the FID components with water. After fully drying the parts, all electrical connections were mechanically cleaned with emery cloth to ensure proper conductivity prior to reassembly.

2.5.4 Chemicals and reagents

Instrument-grade CO_2 (99.99%; Praxair) was employed as the mobile phase. Analyte standards were prepared by dissolving test compounds (all ≥99%; Sigma-Aldrich) in HPLC-grade solvents (Sigma-Aldrich). The specifics of these standards such as composition and concentration are shown in Table 2-4 below. All other details and variations are described in Chapter Seven.

Table 2-4: Composition and concentration of analyte standards employed in Chapter Seven.

Solution	Analyte	Concentration (mg/mL)	Solvent
Citric acid solution	Citric acid	11.94	Water
Acidified citric acid	Citric acid	11.11	pH 1 HCl solution
Acidified DBS	Dodecylbenzene sulfonic acid (DBS)	11.19	pH 0 HCl solution
DBS in CS_2	Dodecylbenzene sulfonic acid	11.05	CS_2
Phospholipid solution	*L*-*α*-Phosphatidylcholine	10.2	Water
Hexanoic acid solution	1-Hexanoic acid	10.62	Water
DBS solution	Dodecylbenzene sulfonic acid	10.74	Water
Stearic acid solution	Stearic acid	10.4	Acetic acid
Oleic acid solution	Oleic acid	10.0	Acetic acid
Light carboxylic acid mixture	Acetic acid 1-Butanoic acid 1-Hexanoic acid	10.51 10.18 10.32	Water
Dodecanoic acid solution	1-Dodecanoic acid	10.17	Methanol

2.5.5 Stationary phases

The various stationary phases employed in Chapter Seven were prepared in HPLC-grade water (2 MΩ cm; Fischer), using high-purity compounds (all ≥99%; Sigma-Aldrich). The specifics of these stationary phases such as composition and concentration are shown in Table 2-5.

Table 2-5: Composition and concentration of stationary phases employed in Chapter Seven.

Stationary Phase	Component	Concentration (mol/L)
pH 1 HCl	Concentrated hydrochloric acid	1 % (v/v)
pH 0 HCl	Concentrated hydrochloric acid	10 % (v/v)
pH 12 NaOH	Sodium hydroxide	0.01016
1.0 M KNO_3	Potassium nitrate	1.004
0.1 M KNO_3	Potassium nitrate	0.1025
0.01 M KNO_3	Potassium nitrate	0.01025
0.1 M $AgNO_3$	Silver nitrate	0.09987
0.1 M DBS	Dodecylbenzene sulfonic acid	0.09646
0.01 M DBS	Dodecylbenzene sulfonic acid	0.009646
0.001 M DBS	Dodecylbenzene sulfonic acid	0.0009646
0.0001 M DBS	Dodecylbenzene sulfonic acid	0.00009646

Chapter Three: **CARBON DIOXIDE MODIFIED SUBCRITICAL WATER CHROMATOGRAPHY**

3.1 Introduction

In order to maximize the solvent strength in SWC, researchers are constantly pushing the thermal boundaries of the stationary phase which can have negative consequences. For example, while eluting compounds from a polymeric column in our lab, significant column damage occurred while operating above 220 °C. As shown in Figure 3-1, the consistent baseline resolution between methanol and ethanol was essentially destroyed after exposure to excessively high temperatures. Such findings in this temperature range are very common in SWC.[103]

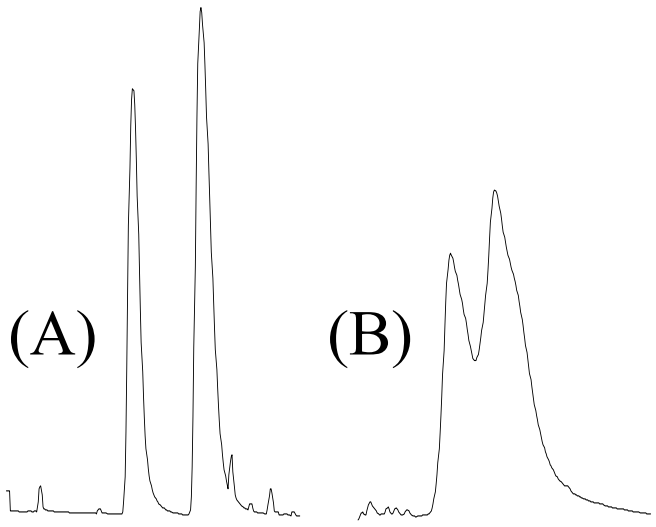


Figure 3-1: Conventional SWC separation of methanol and ethanol on a 150 mm × 2.1 mm I.D. (5 µm) poly(styrene-divinylbenzene) column using 200 µL/min of pure water at 70 °C (A) before and (B) after thermally damaging the column by exposure to temperatures above 220 °C.

In recent years however, the SWC mobile phase has been modified by blending non-polar co-solvents into the water, which reduces the overall mobile phase polarity in SWC without requiring extremely high temperatures. For example, by using a DMSO (dimethyl sulfoxide) / water mobile phase in SWC, the operating temperatures were successfully reduced and considerably non-polar analytes were eluted.[111] Similar approaches have also been demonstrated in SWC employing acetonitrile[39] and methanol.[112] However, in reality these approaches mimic conventional HPLC rather than SWC. Further, aside from the environmental benefit of purely aqueous SWC, the unique ability to use the FID in SWC is critical for many users. Unfortunately, DMSO, methanol and acetonitrile respond in this detector and prohibit use of the FID with these techniques. Therefore, a method that could facilitate the elution of non-polar analytes in SWC while maintaining the conventional benefits of environmental and FID compatibility would be useful. For example, other studies have added polar components such as formic acid[66] and ammonia[65] and pH modifiers[38] such as phosphate, carbonate, and borate to the mobile phase in efforts to alter the selectivity of polar analytes in SWC separations. Even though the additives were eventually found to cause considerable system plugging and corrosion over the long term, their unresponsive nature in the FID did allow the detector to be used in such SWC applications.

A non-polar modifier that has never been explored in SWC is CO_2, perhaps owing to the relative immiscibility that exists between water and CO_2. However, due to the higher temperature subcritical conditions employed in SWC, such an approach may allow for increased solubility of these relatively immiscible solvents and improve the elution of non-polar analytes in SWC. For example, in SWC the mobile phase often has a polarity

nearing that of methanol, which is soluble in CO_2. Therefore, a region of improved solubility may be attainable. Further, CO_2/water mobile phases have the potential to offer a relatively „green' separation medium, devoid of organic solvents, that is transparent to the FID.

This chapter describes blending non-polar CO_2 with subcritical water for the first time as a mobile phase in SWC. The characteristics of this method are presented and discussed. As well, direct comparisons are made between it and conventional purely aqueous SWC separations in order to assess the relative utility of this new technique. The instrumental parameters described in section 2.1 are used in these experiments.

3.2 Mobile Phase Characterization

Initial efforts focused on optimizing the CO_2/water mobile phase for SWC separations. It was found that at lower temperatures (below about 80 °C) peak shapes of 1-butanol were often distorted and frequently appeared as doublets. This is shown in Figure 3-2A for a 1-butanol test analyte with a CO_2 / water mobile phase at 20 °C. The peak appearance is indicative of relatively poorly mixed mobile phase components, which is reasonable in view of the reduced solubility of liquid CO_2 in water at room temperature.[113] However, at higher temperatures the doublet profiles disappeared and peak appearance improved dramatically as shown in Figure 3-2B and Figure 3-2C. This suggests better mobile phase mixing of the two components and is consistent with other studies that reported significantly increased solubility of sub and supercritical CO_2 in high temperature water.[114] (Still, it should be noted that the opposite trend has been observed for CO_2 and water mixtures at much lower temperatures and pressures.[115])

Thus, analogous to high temperature water in SWC increasing its affinity for non-polar analytes, it also appears that the mobile phase solubility of non-polar CO_2 can also increase under such conditions. Overall then, the chromatographic trends of Figure 3-2 reproduced for other analytes and it was generally found that the mixed mobile phase system should be operated at a minimum temperature of about 80 °C to avoid peak splitting.

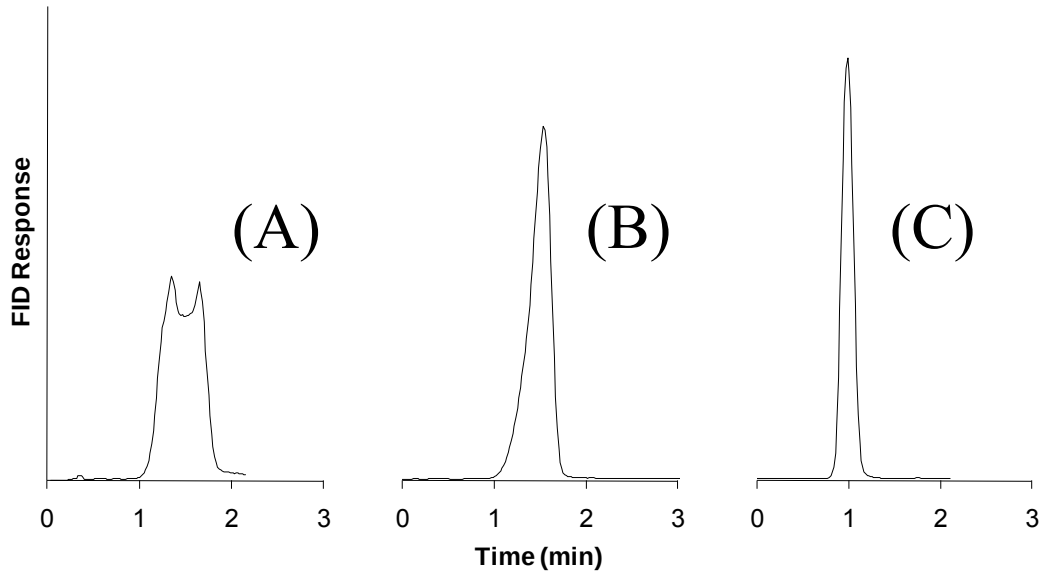


Figure 3-2: CO_2 / water elution of a 1-butanol test analyte (60 mg/mL) at (A) 20 °C, (B) 100 °C and (C) 150 °C. The water flow rate is 200 µL/min and the CO_2 pressure is 160 atm with a 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column.

3.3 Retention Control

In conventional SWC, temperature controls mobile phase polarity and therefore analyte retention. While this is also true for the blended mobile phase described here, it

was additionally found that CO_2 pressure could also impact retention through altering mobile phase composition and polarity analogous to a temperature increase. This is demonstrated for 1-butanol in Figure 3-3. As seen, retention is not only reduced for 1-butanol when temperature is increased from 100 °C to 150 °C, but it is also effectively reduced as CO_2 pressure is increased across each isotherm. Of note, the impact on 1-butanol retention of increasing from 100 °C to 150 °C (with 80 atm of CO_2 and 200 µL/min of water) can also be achieved by about a 50 atm increase in CO_2 pressure isothermally at 100 °C. Thus, increasing the amount of CO_2 present can add another dimension of retention control in this SWC system. Such a parameter could be useful, for

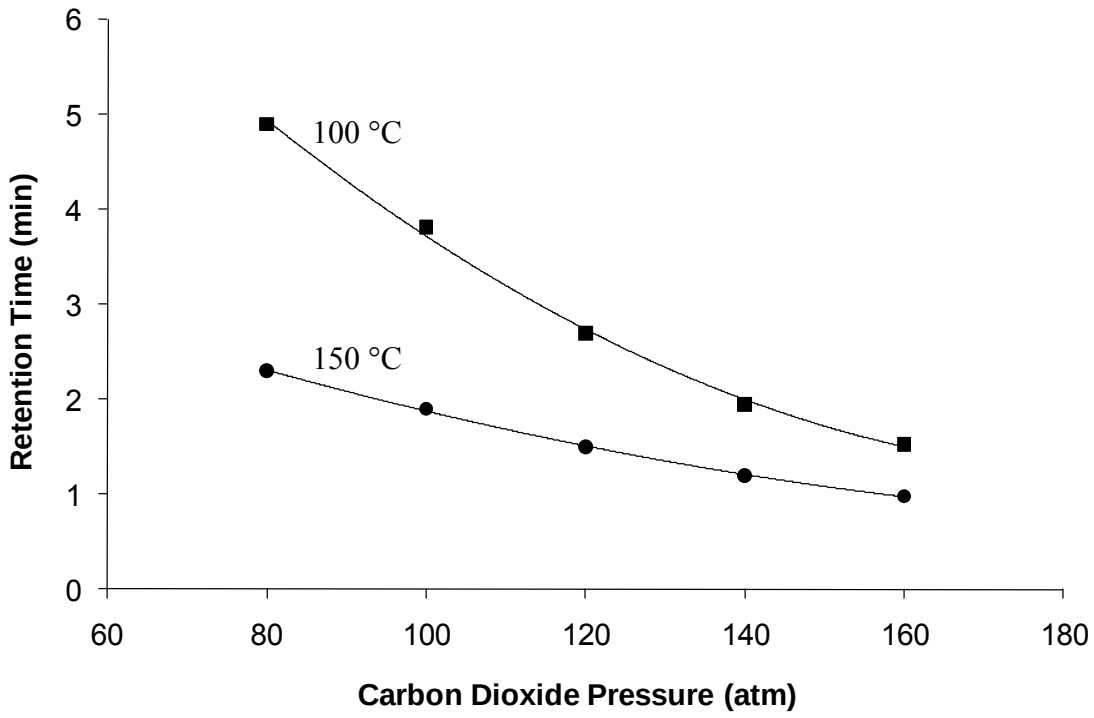


Figure 3-3: 1-Butanol retention as a function of CO_2 pressure at 100 °C (■) and 150 °C (●). 200 µL/min water, 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column.

example, in the separation of more thermally labile analytes or column packings in SWC that normally cannot withstand the high temperatures conventionally used in this method. However, one aspect of such an observation that must clearly be addressed is the impact of CO_2 pressure on column flow rate.

For instance, both temperature and pressure can alter the CO_2 flow rate. Typically, at 100 °C with CO_2 pressures of 100 to 250 atm, the resulting flow can vary from about 100 to 850 μL/min. At 150 °C, over the same pressure range, the flows further increase by about 100 μL/min or so above these values. Therefore, since increases in column flow rate can impact retention time, it is useful to investigate to what extent flow rate influences analyte retention relative to the change in mobile phase composition occurring due to the presence of CO_2. Figure 3-4 illustrates this for 1-butanol in both pure water and in a blended CO_2 / water mobile phase at different CO_2 pressures and hence flow rates. As can be seen, retention does decrease as the pure water flow rate increases. However, by comparison, for the exact same temperature and total column flow rate the CO_2 / water system greatly reduces retention. For example, as depicted by the vertical gap between the two curves in Figure 3-4, it is observed that at 100 °C and a constant total column flow rate of 400 μL/min the retention time of 1-butanol drops nearly 4-fold between the conventional SWC system and the blended CO_2 / water system. Thus, the presence of non-polar CO_2 in the mobile phase has a relatively considerable influence on retention independent of column flow rate alone. Figure 3-5 demonstrates how the CO_2 mobile phase composition can be used to control analyte retention with a test mixture of short chain carboxylic acids under isothermal conditions. As can be seen, as the CO_2

pressure is increased by 60 atm, the sample elution time reduces by a factor of about three.

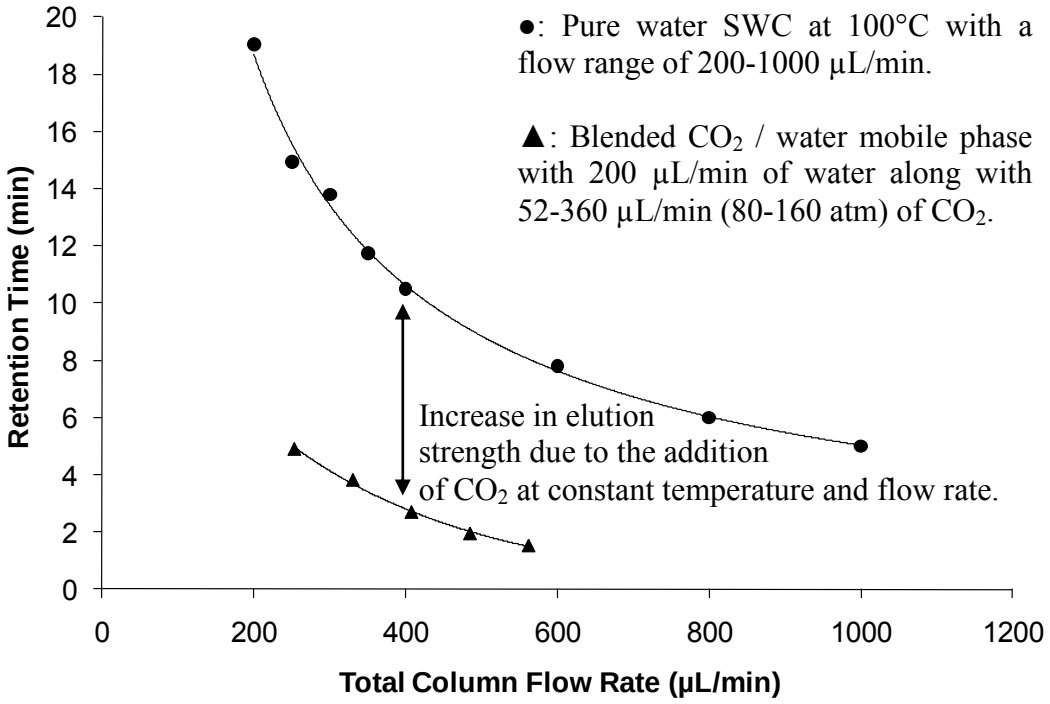


Figure 3-4: 1-Butanol retention as a function of total column flow rate for a pure water (●) and a CO_2 / water (▲) mobile phase. 100 °C and a 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column for both. The vertical gap depicts the isothermal impact on retention of adding CO_2 to the mobile phase at a constant total column flow rate.

3.4 Comparisons with Conventional SWC

In order to better evaluate the relative elution strength of the blended CO_2 / water system for various solutes, it was directly compared in SWC separations using a pure

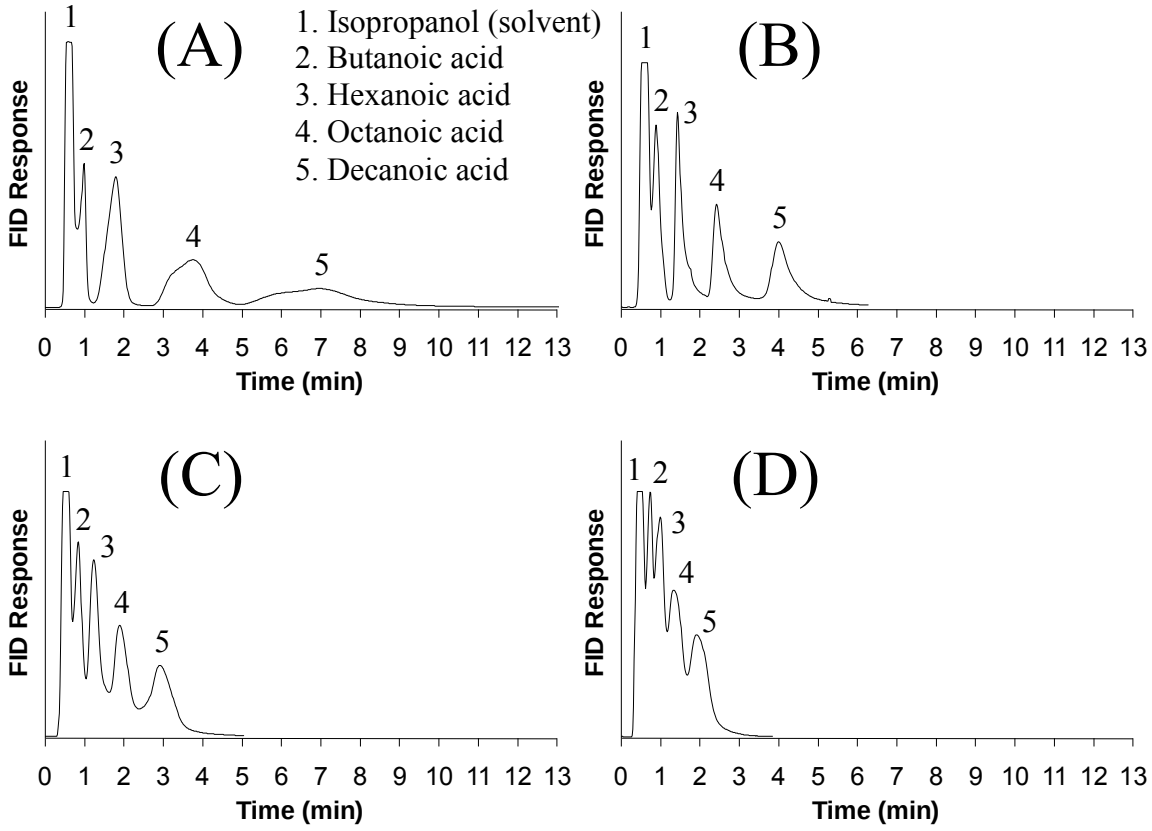


Figure 3-5: CO_2 / water SWC separation of short chain carboxylic acids at 150 °C, 200 µL/min of water and CO_2 pressures of (A) 180 atm (560 µL/min), (B) 200 atm (620 µL/min), (C) 220 atm (720 µL/min) and (D) 240 atm (800 µL/min). 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column.

water mobile phase. Figure 3-6 shows some analytes with moderate to large retention in conventional SWC at the most aggressive condition of 200 °C.[22,30,36,42,65,72] As seen, many of the analytes are either late-eluting or do not even appear within the 60 minute chromatograms. For example, in Figure 3-6A SWC cannot elute 1-octanol after 1 hour at 200 °C. However, in the mixed system at 100 °C with the application of 180 atm (430

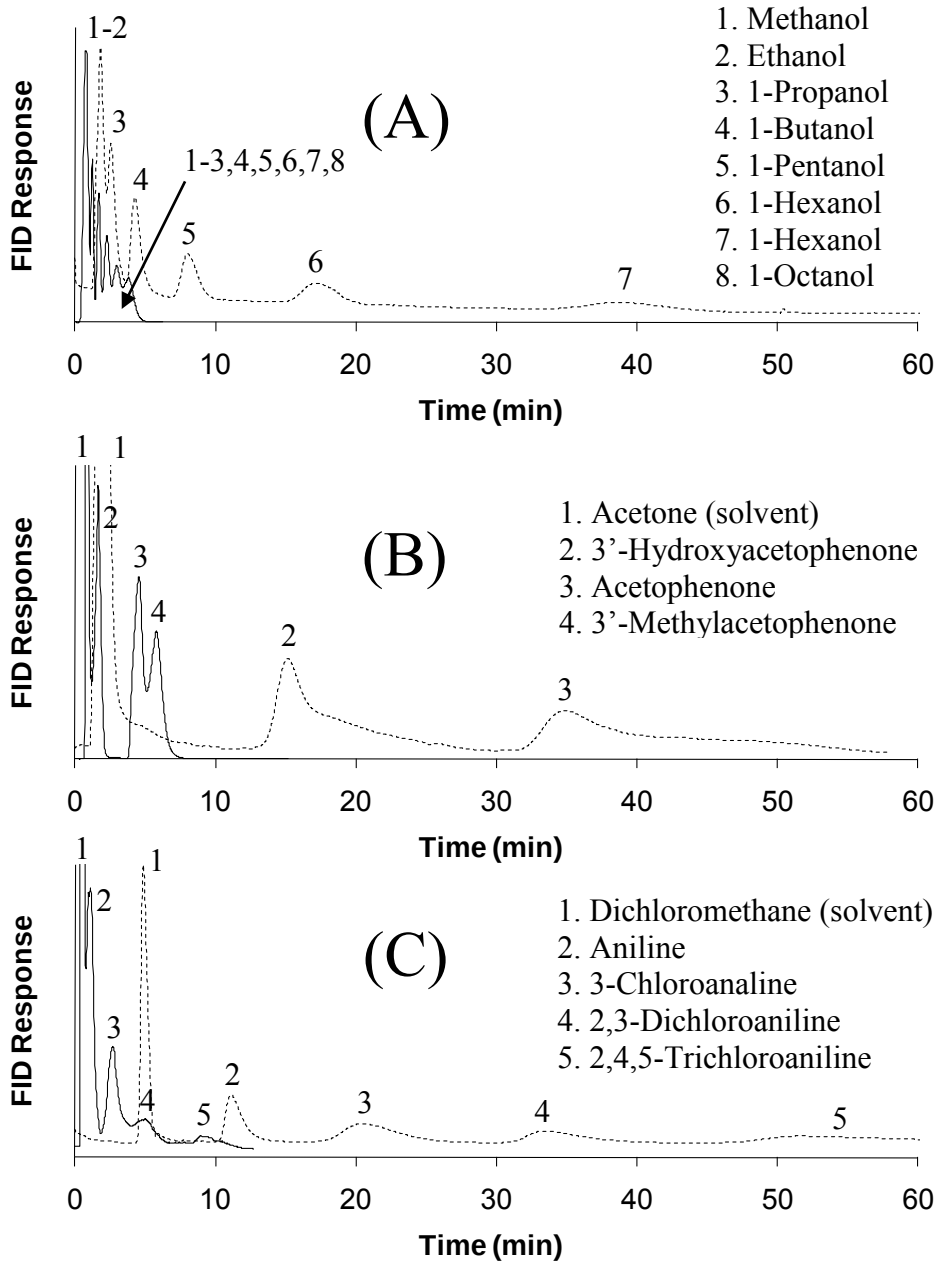


Figure 3-6: Comparisons of conventional water (dashed line) and CO_2 / water (solid line) SWC separations. All trials used 200 µL/min of water. Conventional SWC was operated at 200 °C. Mixed mobile phase conditions were 180 atm (430 - 450 µL/min) CO_2 at 100 °C (A and B) and 220 atm (820 µL/min) at 150 °C (C). 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column.

μL/min) of CO_2 it appears in less than 4 minutes. Also, as seen in Figure 3-6B, a conventional SWC separation of acetophenone derivatives showed no elution of 3'-methylacetophenone after 60 minutes at 200 °C, while those analytes that did elute were relatively broad and tailed in appearance. In contrast to this, the mixed system at 100 °C and 180 atm (450 μL/min) of CO_2 was able to elute all three components in less than six minutes with good peak shapes. Finally, Figure 3-6C shows a separation of aniline derivatives where the final tri-chloro species elutes at around 52 minutes using a pure water mobile phase at 200 °C. By comparison, the mixed mobile phase system at 150 °C with 220 atm (860 μL/min) of CO_2 elutes all four components within 10 minutes.

Next, similar experiments were performed for considerably less polar analytes which are very highly retained in conventional SWC.[42,62,111] For example, Figure 3-7A shows that in conventional SWC only benzene of a benzene, toluene, ethylbenzene, and *p*-xylene (BTEX) mixture is eluted within 1 hour at 200 °C, but with a considerably broad peak profile. Comparatively, the mixed system eluted all of the BTEX peaks in less than six minutes at 100 °C and 160 atm (270 μL/min) of CO_2. This elution strength compares favourably to reversed phase HPLC which eluted a BTEX mixture in about 12 minutes at 25 °C and using an 80% DMSO in water mobile phase.[111] Further, even 66% DMSO in water was required at 125 °C to produce the latter result in high temperature HPLC.[111] Finally, with regard to conventional SWC, while highly retained on a PRP-1 column at 200 °C, the BTEX sample can be eluted from a C18 column in approximately 45 minutes at 200 °C.[42] Thus, the current system compares favourably with other methods.

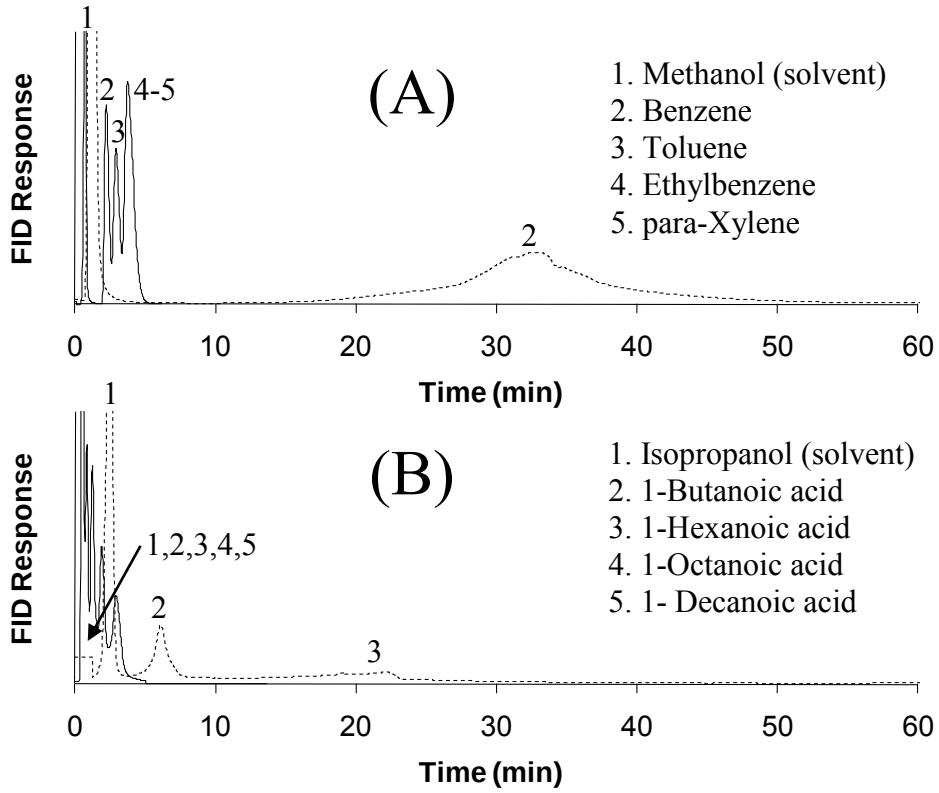


Figure 3-7: Comparisons of conventional water (dashed line) and CO_2 / water (solid line) SWC separations. All trials used 200 µL/min of water. Conventional SWC was operated at 200 °C. Mixed mobile phase conditions were 160 atm (270 µL/min) and 100 °C (A); 220 atm (720 µL/min) and 150 °C (B). 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column.

In Figure 3-7B, a separation of short chain carboxylic acids showed that only butanoic and hexanoic acids eluted within 60 minutes at 200 °C but with increasingly poor peak shape. Conversely the CO_2 / water system eluted all four components in less than four minutes with good peak shapes at 150 °C and 220 atm (720 µL/min) of CO_2. Figure 3-8A shows a separation attempt of free fatty acids where no analyte elution was

observed after 1 hour at 200 °C in conventional SWC. With the mixed system however, all of the analytes eluted in less than 10 minutes at 150 °C and 260 atm (875 µL/min) of CO_2. Lastly, Figure 3-8B depicts the separation of the alcohol ethoxylate polyoxyethylene (n~2) cetyl ether (Brij®-52). Under conventional SWC conditions, no peak elution was observed over 60 minutes at 200 °C. However, the mixed system eluted all four major components in about 10 minutes at 150 °C and 260 atm (800 µL/min) of CO_2. It is important to point out that these latter two non-volatile solutes are difficult to

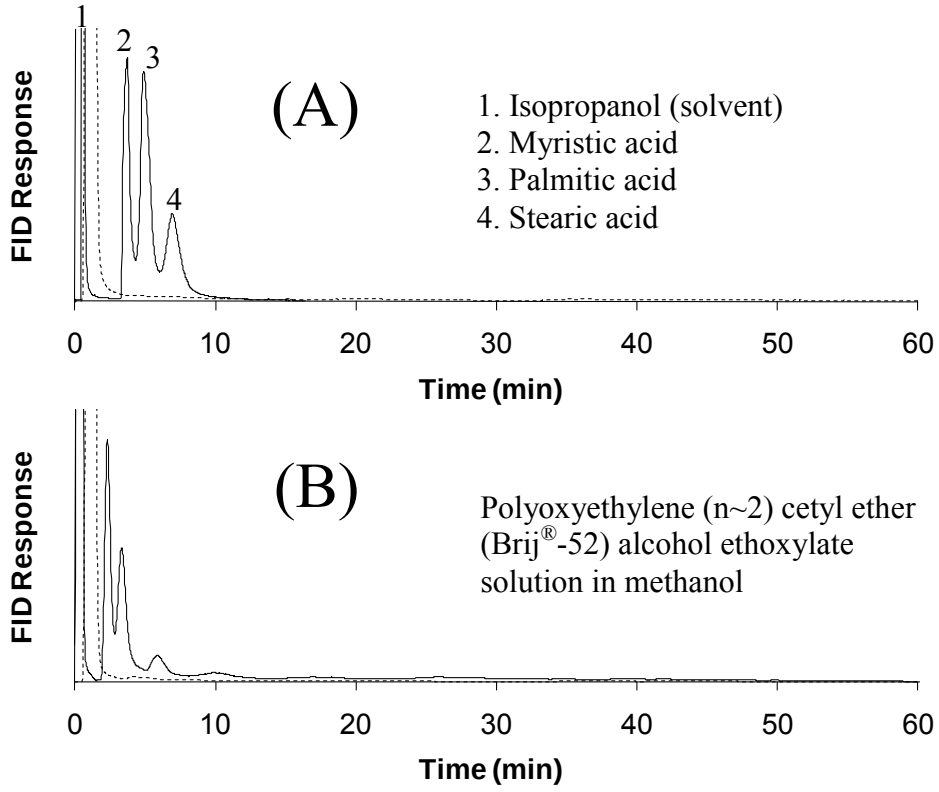


Figure 3-8: Comparisons of conventional water (dashed line) and CO_2 / water (solid line) SWC separations. All trials used 200 µL/min of water. Conventional SWC was operated at 200 °C. Mixed mobile phase conditions were 260 atm (800 - 875 µL/min) and 150 °C. 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column.

analyse by GC without prior derivatization. Additionally, alcohol ethoxylates can be challenging in HPLC analysis as well due to the lack of a strong chromophore for UV-Vis detection.[116] Overall then, the results of the non-polar analyte separations in Figure 3-6, Figure 3-7, and Figure 3-8 indicate that the mixed mobile phase system shows a significant increase in elution strength for non-polar analytes compared to conventional SWC. It is worth reiterating that Figure 3-6, Figure 3-7, and Figure 3-8 are only intended to demonstrate this improvement in elution strength. As such, it should be pointed out that the ability to generate better resolved chromatograms is possible through reducing the elution strength as demonstrated in Figure 3-5.

3.5 More Challenging Separations

Non-volatile polyethylene glycols (PEGs), with average molecular weights of 200, 400, 600 and 1000 g/mol were also explored in the mixed system. PEGs were selected as test analytes since they pose a separation challenge in conventional forms of chromatography. For example, GC cannot be employed for all but the lowest molecular weight PEG variants due to their low volatility.[117] In addition, HPLC analysis is troublesome since PEGs require derivatization in order to be detected in a UV-Vis detector.[118]

The PEG 200 solution was successfully analysed using conventional SWC operated at 150 °C with the elution and separation of the substituent components in less than 10 minutes. However, difficulties arose in SWC as the PEG molecular weight was increased. At 200 °C, SWC was able to elute and separate the PEG 400 analyte mixture in about 40 minutes with considerable broadening of the later-eluting peaks. Finally,

conventional SWC could not elute all of the PEG 600 mixture at 200 °C in 60 minutes. Since difficulties were encountered with PEG 600, PEG 1000 was not attempted in conventional SWC.

By comparison, the mixed mobile phase system successfully eluted all four PEG mixtures. For example, Figure 3-9A shows the decrease in the elution time of PEG 600 over SWC when employing the mixed system. At 200 °C in conventional SWC, some of the PEG components were observed in 60 minutes with poor peak shape. Conversely, at a relatively mild 160 atm and 100 °C, the mixed system rapidly eluted all of the PEG components. Figure 3-9B demonstrates the ability of the mixed mobile phase system to elute the higher molecular weight solution, PEG 1000. As seen, although tailing and somewhat noisy in appearance, the system is able to elute this rather large polymer where elution in conventional SWC appears impossible.

However, as observed in Figure 3-9, it should be noted that no separation of the individual PEG components was obtained. When conditions were altered to more highly retain the PEGs, the individual components were still not resolved and the PEG peak began to tail appreciably. Therefore, at this time, it appears that the column used in the mixed mobile phase system is not able to separate the individual PEG polymers. In addition, as mentioned above, system noise was evident when separating PEGs. The noise was likely due to restrictor erosion and could readily be minimised by replacing the system restrictor.

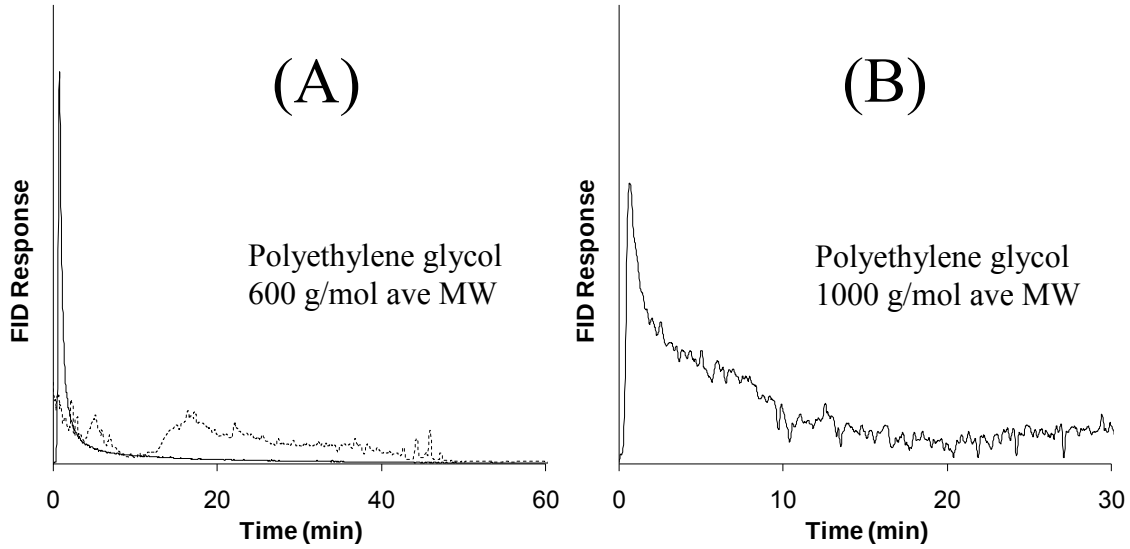


Figure 3-9: Comparisons of conventional water (dashed line) and CO_2 / water (solid line) SWC separations of PEG solutions. All trials used 200 µL/min of water. Conventional SWC was operated at 200 °C. Mixed mobile phase conditions were 160 atm (500 µL/min) and 100 °C (A), 260 atm (950 µL/min) and 150 °C (B). 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column.

A mixture containing methionine and four oligopeptides (Gly-Tyr, Val-Tyr-Val, Tyr-Gly-Gly-Phe-Met and Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) was also explored in the mixed mobile phase system. In this case, significant problems were encountered from the outset. A successful separation was not obtained in either conventional SWC or in the mixed mobile phase system. At 200 °C, conventional SWC resulted in a single peak with considerable tailing, likely due to analyte degradation (Figure 3-10). This observed decomposition at elevated temperatures is consistent with other studies that have shown peptides to denature under high temperature conditions.[119] Conversely, when operated at

more mild conditions (i.e. to prevent analyte degradation), conventional SWC lacked the solvating strength to elute these compounds.

The most successful separation in the mixed system is shown in Figure 3-10 and was performed at 70 °C with 140 atm of CO_2. In this case, one major analyte peak was observed in about 20 minutes with possibly a second peak shouldered on the solvent. The major peak was considerably broadened, which again could possibly be due to analyte thermal degradation or poor separation on the PRP-1 column used. When attempts were made to perform lower-temperature separations in the mixed system (200 atm, 50 °C),

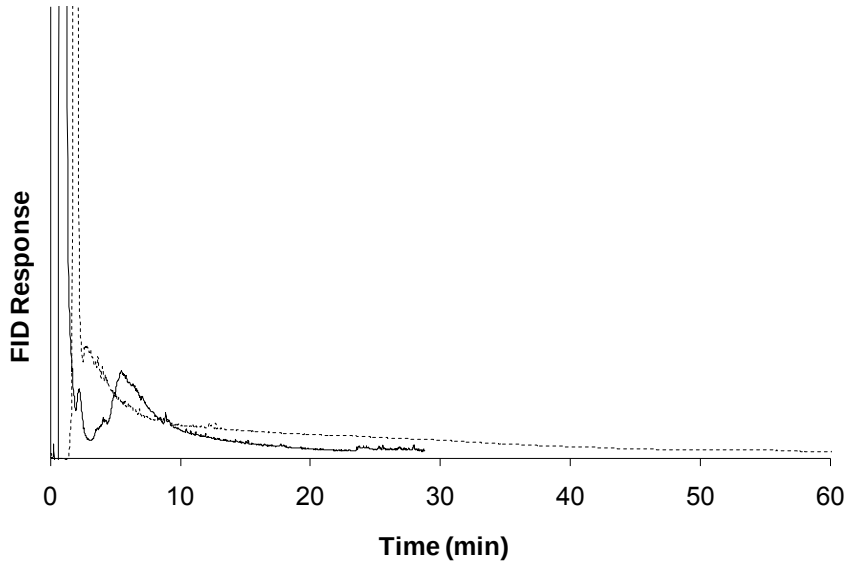


Figure 3-10: Comparison of conventional water (dashed line) and CO_2 / water (solid line) SWC separations of a peptide mixture (five components) in methanol. Each used 200 µL/min of water. Conventional SWC was operated at 200 °C. Mixed mobile phase conditions were 140 atm (200 µL/min) and 70 °C. 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column.

dramatic peak splitting was observed which is consistent with the behaviour previously demonstrated in Figure 3-2. Therefore, at this time, it appears that the column used with this mixed mobile phase system is unable to successfully separate these peptides.

3.6 Gradient Programming

In order to see the influence of gradients in the mixed mobile phase system, both temperature and composition programming were explored. Figure 3-11A shows a typical result of a temperature programmed separation of short chain carboxylic acids with 180 atm of CO_2 added to the aqueous mobile phase. As can be seen, the temperature program generates decent peak shapes without any major deleterious effects. Conversely, Figure 3-11B shows the typical results of CO_2 pressure programming in the mixed system for a mixture of carboxylic acids. As can be observed in this figure, the isothermal CO_2 pressure program can also effectively elute the various test analytes. However, as shown, relative to the temperature programmed trials some irregularities in peak shape were frequently observed during pressure programmed separations. While the cause of this is currently unknown, it may perhaps be due to inadequate time allotted for the mobile phase to pre-heat and equilibrate during the length of the program. If so, increasing the pre-heating residence time of the mobile phase in the oven may improve this, since thermal delays in SWC heating have been reported.[72] However, more work is needed to determine the extent to which this might be effective. As well, consistent with the findings of Figure 3-3 and Figure 3-4, we also often found CO_2 pressure gradients to have a relatively greater influence on analyte retention in the mixed system than temperature programming alone. Thus, temperature and pressure gradient programming appear

potentially useful in the mixed mobile phase system and some further optimization of these techniques would still be beneficial.

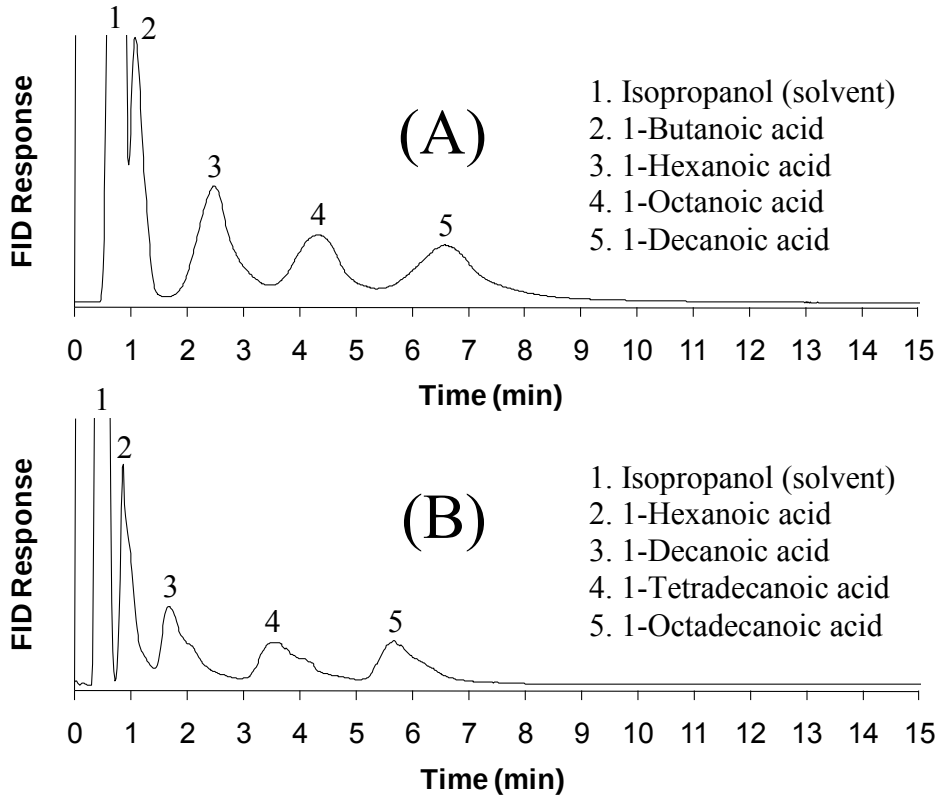


Figure 3-11: Temperature (A) and CO_2 pressure (B) programming in the mixed mobile phase system. All trials used 200 µL/min of water. In (A) 180 atm is used with a temperature program of 80 °C initially for 1 minute followed by a 16 °C/min rise to 150 °C. In (B) a 150 °C temperature is used with a CO_2 pressure program of 240 atm initially for 3 minutes then a 13 atm/min increase to 280 atm. 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column.

It should be noted that total miscibility of CO_2 and water should not be inferred from any of the above work, since this is very unlikely. Currently, while the state of the

CO_2 / water mobile phase within the column is uncertain, it could perhaps be that a microemulsion-like phase is forming such as those employed in other modes of chromatography.[120] However, further work is needed to verify this. Nonetheless, the impact of CO_2 in the mobile phase and its effectiveness in separations is observed.

Further, the FID performed well under these conditions as would be expected. For instance, previous SWC studies have found the response of the FID with a pure water SWC mobile phase to be within an order of magnitude of optimal GC conditions.[31] Also, it has been found that the sensitivity of the FID with a pure CO_2 mobile phase is within a factor of three to that of GC-FID.[121] Accordingly then, the FID displayed no unusual characteristics with the blended CO_2 / water mobile phase.

3.7 Column Recovery

On a final note, as demonstrated in Figure 3-1, often while working in SWC the thermal limits of the separation column can be constantly tested, which sometimes results in the loss of column efficiency. Indeed, such findings have assisted in establishing the thermal limits for various stationary phases in the SWC literature.[103] For example, as noted earlier we have observed a dramatic reduction in separation performance after exceeding 200 °C on a polymeric PRP-1 column.[72] Since manufacturers recommend column operation at no greater than 80 °C, it is often assumed that degradation of the stationary phase has occurred in such instances and these columns are normally then discarded. In the course of this work, a column that was previously damaged by excess heating was inadvertently used. Subsequently, it was actually found that back-flushing pressurized CO_2 at 100 °C followed by a slow descent to room temperature reclaimed

much of the lost efficiency. Note that in HTLC the same observed efficiency loss is instead attributed to thermal expansion of the stainless steel column.[12] For instance, a large spike in temperature can increase the column diameter to the extent that it allows movement of packing particles inside the column. This shift away from packing uniformity in turn results in decreased efficiency. In this regard, columns used at high temperature must be packed very tightly or packed at high temperatures to avoid the unsettling of the particle bed.[119]

However, back-flushing high-pressure CO_2 through the thermally damaged PRP-1 column (Figure 3-12A) appears to reverse some of the lost efficiency and improves separations. For example, as seen in Figure 3-12B, after treatment with pressurized CO_2, the separation was notably improved and approximately half of the lost resolution was regained as a result. The treatment procedure involved back-flushing the column with 260 atm CO_2 at 100 °C for 15 minutes followed by a negative temperature program (-12 °C/min) to room temperature where it was held for a further 15 minutes. It is believed that this causes shuffling of the stationary phase particles back toward optimal packing, thereby filling in some of the gaps created by the thermal expansion of the column diameter. This procedure may therefore be of use to SWC (and perhaps even HTLC) users experiencing similar difficulties. Particularly, since it has been noted that the development of a column regeneration procedure with a non-polar eluent could further advance SWC.[101] It is currently unknown if other solvents besides CO_2, or a more rapid temperature descent could achieve the same results.

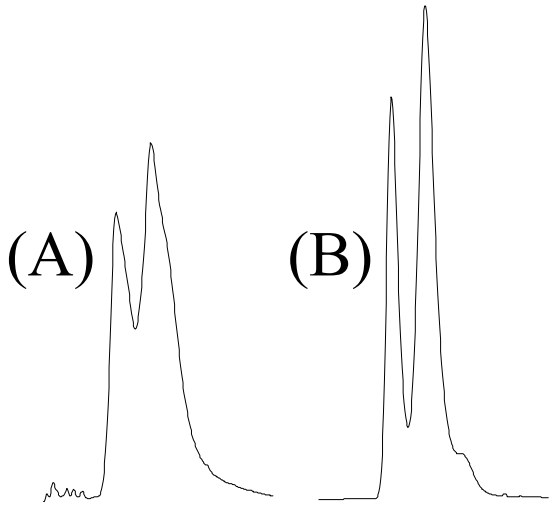


Figure 3-12: Conventional SWC separation at 200 µL/min and 70 °C of methanol and ethanol on a 150 mm × 2.1 mm I.D. (5 µm) PRP-1 column after thermally damaging the column (A). Trace (B) shows the same separation after conditioning the column with 260 atm of CO_2 at 100 °C for 15 minutes followed by a slow decrease (-12 °C/min) to 20 °C and holding for 15 minutes.

3.8 Conclusions

Through the addition of non-polar CO_2 to conventional purely aqueous SWC, the polarity of the mobile phase can be significantly decreased. As such, this greatly extends the ability of SWC to analyse non-polar analytes while maintaining the primary features of environmental and FID compatibility that SWC is known for. The mixed CO_2 / water system also appears amenable to dual gradient programming modes using temperature or CO_2 pressure to systematically alter mobile phase polarity in SWC separations. Further, the addition of CO_2 reduces the operating temperatures required to elute analytes

separated in conventional purely aqueous SWC. Therefore, such a feature may potentially facilitate the use of stationary phases previously precluded from use in SWC due to the high temperatures required by the method.[103] As well, this feature may allow for the analysis of thermally labile analytes which have previously been incompatible with SWC due to thermal instability issues.

Chapter Four: **EXPLORING A MODEL PHARMACEUTICAL WITH SUBCRITICAL WATER CHROMATOGRAPHY AND CARBON DIOXIDE MODIFIED SUBCRITICAL WATER CHROMATOGRAPHY**

4.1 Introduction

Historically, employing alternative solvent systems such as SWC to separate pharmaceutically interesting compounds has been troublesome due to the thermally labile nature of many such compounds. For example, the elevated temperatures of HTLC have proven difficult for proteins which rapidly decompose above 80 °C.[119] The authors of this study did state that protein separations were possible above 120 °C if the proteins were eluted sufficiently fast (i.e. under 1 min). However, this timeframe is unrealistic for the proper HPLC separation of a protein mixture. Other HTLC studies have demonstrated steroids to be stable to 120 °C[54] while a separation of metabolites proved challenging due to the decomposition of diglucuronide at 120 °C.[122] In addition to a temperature effect alone, the stationary phase seems to play a role in high temperature stability of analytes as well. For example, sterols[123] and thalidomide[124] both readily decompose on graphitic carbon-clad zirconia columns under HTLC conditions while the former was stable on an octadecyl-bonded silica (ODS) column and the latter was stable on poly(styrene-divinylbenzene) polymeric column.

Since SWC also often requires very high temperatures to achieve greater solvent strength, it has encountered mixed success in the realm of pharmaceutically relevant separations. Successful separations have been demonstrated for the analysis of steroids at 160 °C[47], the anti-cancer drugs 5-fluorouracil, chlorambucil, and melphalan at 160 °C[46], analgesics such as caffeine and phenacetin at 190 °C[41], barbiturates at 200 °C[64], and the

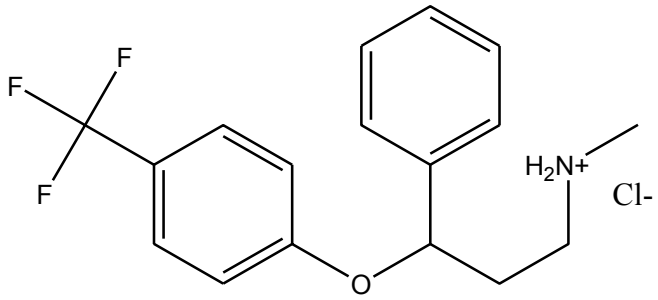
vitamins pyridoxine (vitamin B6) and riboflavin (vitamin B1) at 200 °C.[56] Challenges are likely more prevalent since an approximately equal number of non-successful separations have appeared in the literature. In this regard, it can be surmised that many more non-successful separations were attempted but never made it into the literature. Published examples of less-than successful attempts in SWC include diuretics which have shown notable degradation at 200 °C[55], acetylsalicylic acid (analgesic) which decomposed at 190 °C[41], and thiamine (vitamin B3) which was unstable above 160 °C.[56] Further, while acetaminophen (analgesic) and aminobenzoic acids (vitamin precursors) have been shown to be compatible in SWC at 60 °C, they both decomposed at column temperatures around 180 °C.[61] In addition to the thermal instability of the analyte of interest, other factors seem to affect some SWC separations. One such example demonstrated a change in analyte ionization above 100 °C for a group of antibiotic sulphonamides which made SWC separations of these molecules challenging.[38]

Since CO_2 modified SWC has been shown to significantly increase the eluent strength in SWC while reducing the operating temperature[108], this environmentally-friendly mode of chromatography may be applicable to separate such thermally labile analytes which are impossible to elute from conventional SWC. Further, since CO_2 modified SWC has been shown to elute compounds significantly faster than conventional SWC, the residence time for an analyte on the column is appreciably decreased. Due to the fact that studies have shown that reducing the residence time of an analyte on the column diminishes the problem with analyte stability[125], CO_2 modified SWC may be able to operate above the thermal maximum of a select analyte while successfully eluting it before it has a chance to denature. Overall, the successful implementation of CO_2

modified SWC to a pharmaceutical separation could show potential of this method to reduce the organic solvent consumption of quality control laboratories in this industry.

4.2 Selection of a Model Pharmaceutical

Fluoxetine hydrochloride, the generic pharmaceutical name of Prozac® or (+)-N-methyl-3-phenyl-3-[(*α,α,α*-trifluoro-*p*-tolyl)oxy]propylamine hydrochloride (Figure 4-1) was selected as a model analyte for a multitude of reasons. Fluoxetine hydrochloride (simply referred to herein as fluoxetine) was first described by Eli Lilly and Company in 1974 as a selective serotonin reuptake inhibitor.[126] As such, fluoxetine is currently approved for the treatment of depression, obsessive-compulsive disorder, panic disorder, bulimia nervosa, and bipolar disorder, and, since its approval in 1987, it has become the most prescribed antidepressant drug world-wide.[127] Although separation methods for the analysis of fluoxetine have been developed for HPLC[128-131] and GC[132], no method for the SWC separation of this molecule has been described. Further, since recent stability studies of fluoxetine verify that it does not decompose in hot water at 175 °C for 30 minutes[133], fluoxetine presents a prime candidate for a model pharmaceutical in probing method development in SWC. In this regard, this chapter pursues the development of a SWC and a CO_2 modified SWC separation method for the analysis of fluoxetine in order to explore the possibility of adapting these novel separation techniques to pharmaceutically relevant separations. The instrumental parameters described in section 2.2 are used in these experiments.



pK_a[134]:	10.05
Molecular mass:	345.79 g/mol
Water solubility[135]:	10.76 mg/mL

Figure 4-1: Structure, pK_a value, molecular mass, and water solubility of fluoxetine hydrochloride.

4.3 SWC Method Development for Fluoxetine

Since no method has been described for the SWC analysis of fluoxetine, efforts were first directed toward developing such a separation method. Multiple columns were available to develop the SWC method including a polybutadiene(PBD)-clad zirconia column, a poly(styrene-divinylbenzene) (PRP-1) polymeric column, a cyano-bonded silica column, and a bare silica normal phase column. As such, these columns were evaluated as potential candidates for the stationary phase in a SWC system for the elution of fluoxetine.

4.3.1 Polybutadiene-clad zirconia column

The SWC elution of fluoxetine was first attempted from the PBD-clad zirconia column. This column represents a reversed phase alternative to ODS which is thermally stable up to temperatures of 200 °C. In addition, the PBD phase is considered more hydrophobic than both ODS and PRP-1 stationary phases.[136] Since elevated temperatures

were to be employed, the thermally labile ODS was not selected as a starting point for method development despite its dominance in reversed phase liquid chromatography. A room temperature water elution was first attempted and, as shown in Figure 4-2A, no fluoxetine peak was observed. In order to increase the mobile phase strength, the oven temperature was next increased to 150 °C and fluoxetine was injected again. Although increasing the temperature to 150 °C does increase the elution power of the mobile phase, again no fluoxetine peak was observed at this temperature as shown in Figure 4-2B. Higher oven temperatures and/or longer elution times were not attempted as to stay comfortably within the thermal stability range of fluoxetine (maximum of 175 °C for 30 minutes). Therefore, these experiments suggest that PBD-clad zirconia is too highly retentitive for the timely elution of fluoxetine in SWC.

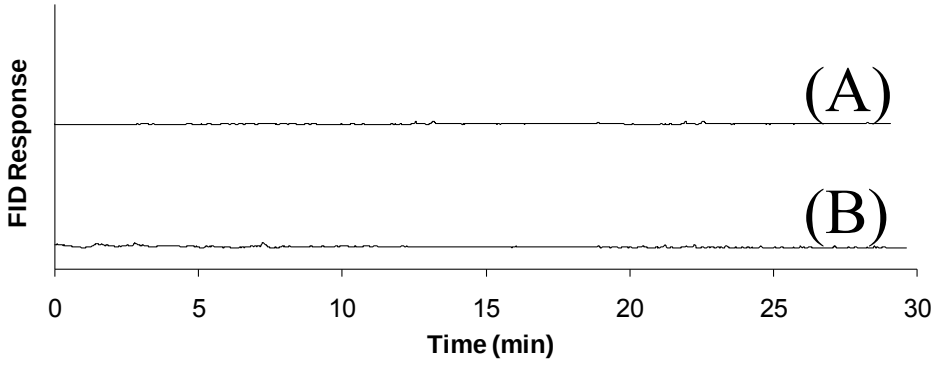


Figure 4-2: Conventional SWC showing no elution of fluoxetine from a 100 mm × 2.1 mm I.D. (3 µm) PBD-clad zirconia column at (A) 20 °C and (B) 150 °C. The water flow rate is 150 µL/min in each case.

4.3.2 PRP-1 column

Since the PBD column appeared too retentitive for the timely elution of fluoxetine in SWC, a PRP-1 column was evaluated next. The PRP-1 phase is less hydrophobic compared to PBD while maintaining a similar thermal ceiling.[103,136] In this regard, it was hoped that SWC would be better able to elute fluoxetine from the PRP-1 column. In order to test this hypothesis, an oven temperature of 20 °C was first attempted (Figure 4-3A) and a small peak was observed eluting at about two minutes. However, upon mass balance comparisons of the area of this peak to the area of a flow injection (i.e. no column present) fluoxetine peak, it is unlikely that this baseline rise is due to the elution of fluoxetine since the peak area is too small relative to the mass injected. Therefore, in attempts to elute fluoxetine from the PRP-1 column, higher oven temperatures of 100 °C and 150 °C were also attempted as shown in Figure 4-3B and Figure 4-3C, respectively. The 100 °C chromatogram again shows the small baseline rise at about two minutes, however at a slightly lower peak area. Again, this baseline perturbation is not strong enough to account for all of the injected fluoxetine mass. The 150 °C chromatogram shows no evidence of this small peak over the entire 30 minutes. Therefore, these trials suggest that SWC is also unable to successfully elute fluoxetine from the PRP-1 column within the constraints of 150 °C and 30 minutes.

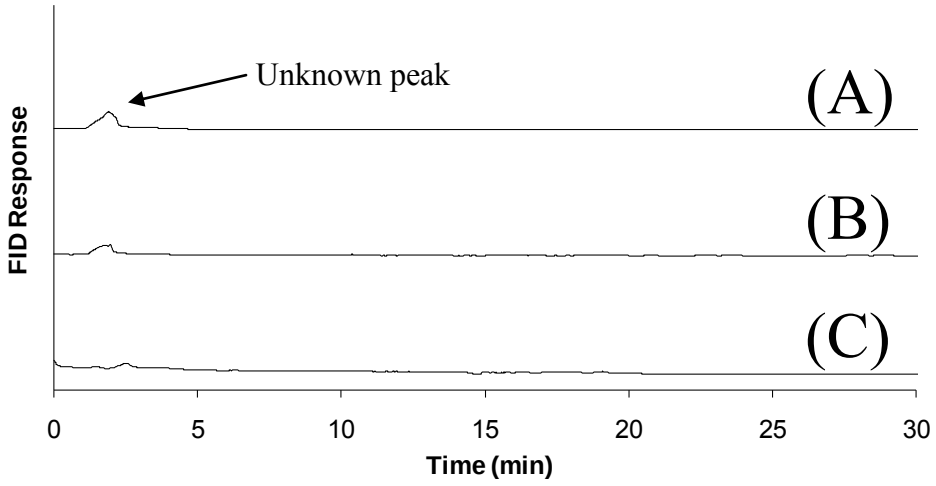


Figure 4-3: Conventional SWC showing no elution of fluoxetine from a 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column at (A) 20 °C, (B) 100 °C, and (C) 150 °C. The water flow rate is 150 µL/min in each case.

4.3.3 Effects of pH

The next attempts to elute fluoxetine from the SWC system involved altering the pH of the mobile phase. Since the HPLC literature often recommends a buffered mobile phase for the elution of fluoxetine[128,130], it was thought that this may aid in the elution of fluoxetine from the SWC system. Reducing the pH of the mobile phase below 10.05 should ensure all of the fluoxetine is in its protonated (i.e. its charged, hydrophilic state) form which should aid in the elution of this molecule. The PRP-1 column was again employed for these studies due to its superior range of pH stability. Trifluoroacetic acid (TFA) and formic acid were selected as mobile phase additives since they both have low response in the FID. Although both contain organic carbon, this carbon is in a highly oxidised state which reduces the FID response to these molecules. The 0.1 % (v/v) TFA

(pH ~2.1) mobile phase was first attempted but, this phase could not provide a smooth baseline and, in turn, blinded the detector. In order to avoid the detection problem, formic acid was next selected and prepared to 0.1 % (v/v) (pH ~2.8). This mobile phase did not respond as strongly in the FID and provided a smooth baseline. Therefore, elution with 0.1 % formic acid was performed at 150 °C (Figure 4-4). However, no appearance of a fluoxetine peak was observed. Again, this experiment supports the suggestion that the PRP-1 column is too retentive for the timely elution of fluoxetine in SWC, even after altering the pH of the mobile phase.

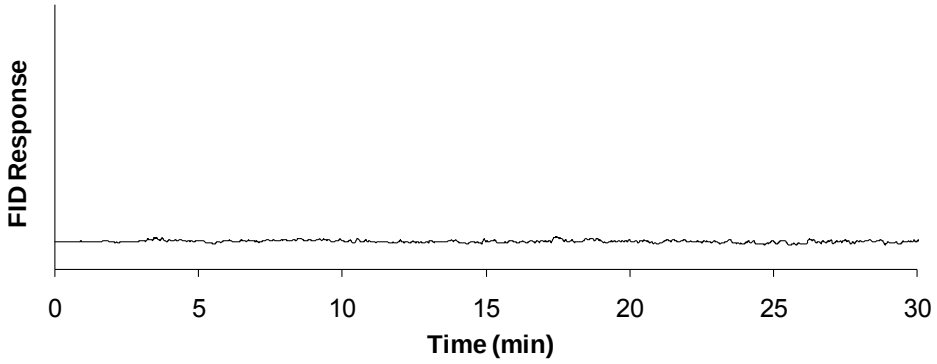


Figure 4-4: Conventional SWC showing no elution of fluoxetine from a 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column at 150 °C with a 0.1 % (v/v) formic acid mobile phase. The mobile phase flow rate is 150 µL/min.

4.3.4 HPLC analysis

Since fluoxetine was not successfully eluted from the SWC system employing PBD and PRP-1 columns, experiments were next performed with hope to shed some insight into the fate of fluoxetine in this system. In this regard, 20 back-to-back injections

of fluoxetine (totalling 107.8 μg) were made onto the PRP-1 column while operating in SWC mode with a pure water mobile phase at 150 °C. As expected, no elution was observed while operating in the SWC mode. However, when this column was removed from the SWC system and placed online in an HPLC chromatograph with a pure acetonitrile mobile phase and UV/Vis detection, a very large response was recorded almost immediately after initiating the mobile phase flow rate. The solid trace of Figure 4-5 shows the elution of this large amount of fluoxetine from the PRP-1 column. The large response confirms that SWC was unable to elute the injected fluoxetine. In addition, the significantly tailed peak profile of fluoxetine might be indicative of some degree of analyte decomposition that has occurred on the column at 150 °C.

For comparison, the dashed trace of Figure 4-5 shows a single injection (5.39 μg) of fluoxetine eluted under the same HPLC conditions. As can be seen in this figure, there is no tailing evident for this smaller injected amount which was eluted at room temperature. The Gaussian profile of this small peak suggests that no decomposition is occurring on the column at 20 °C. In this regard, Figure 4-5 confirms that SWC is unable to elute fluoxetine from the PRP-1 column and may also indicate that some degree of fluoxetine decomposition is occurring at 150 °C in the SWC system. Therefore, these experiments further reinforce the inability of SWC to successfully elute fluoxetine.

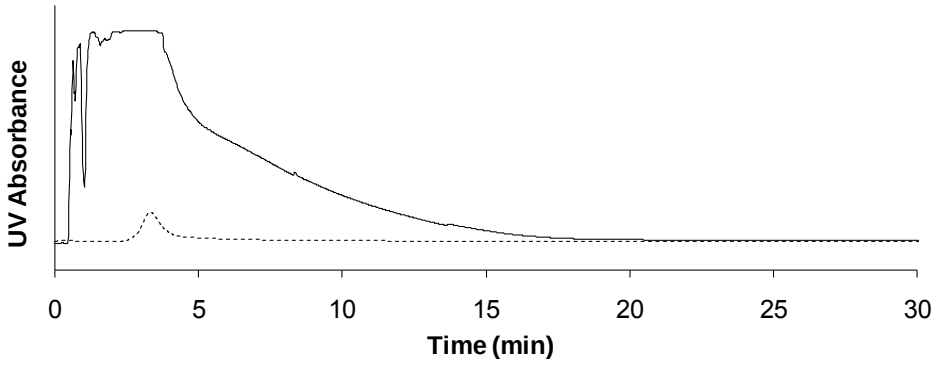


Figure 4-5: HPLC-UV/Vis elution of fluoxetine from a 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column at 20 °C. The acetonitrile mobile phase flow rate is 150 µL/min. The solid trace is the acetonitrile elution profile after loading 107.8 µg of fluoxetine onto the column using a pure water mobile phase. The dashed trace is a single injection (5.39 µg) of a fluoxetine standard eluted with acetonitrile.

4.3.5 Cyano-bonded silica column

Since SWC is unable to elute fluoxetine from both the PBD and the PRP-1 column, an even less hydrophobic phase was next attempted. A cyano-bonded silica phase was selected due to its very low hydrophobicity relative to most other reversed phase HPLC columns.[136] In this regard, SWC separations on cyano columns more resemble traditional normal phase separations. Therefore, since a water mobile phase has very high eluting strength in normal phase chromatography, the combination of a stationary phase with low hydrophobicity and mobile phase with strong eluting power should help the elution of fluoxetine from such a system. A separation at an oven temperature of 20 °C was first attempted, however, as shown in Figure 4-6, no elution of fluoxetine was again observed. Due to this result, higher column temperatures were not

attempted with this column since monomeric-bonded silica phases (such as this cyano-bonded phase) have been found to be very unstable in hot water[103] and the venture appeared unpromising. The lack of fluoxetine elution from this column can perhaps be explained since, although cyano-bonded columns have limited hydrophobicity, they can readily form strong dipole-dipole interactions with charged species and π-π interactions with species that contain π-electron systems.[137] Since fluoxetine satisfies both of these criteria, this could perhaps explain the highly retained nature of fluoxetine on a cyano-bonded column. As such, this column is also not a viable option as a stationary phase for the development of a SWC analysis method for fluoxetine.

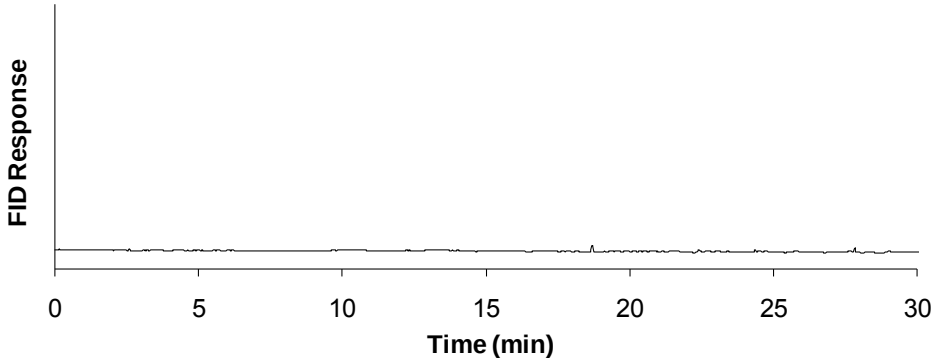


Figure 4-6: Conventional SWC showing no elution of fluoxetine from a 250 mm × 4.6 mm I.D. (5 µm) cyano-bonded silica column at 20 °C with a pure water flow rate of 500 µL/min.

4.3.6 Bare silica packed capillary column

The final column attempted for the elution of fluoxetine in SWC was a bare silica, packed capillary column which was prepared in house. Silica represents a stationary

phase with even less hydrophobicity when compared to the previously attempted cyano-bonded silica phase.[136] In addition, akin to cyano phases, bare silica is a normal phase column and, as such, pure water should prove to be a powerful eluent in this mode. The elution of fluoxetine was first attempted at a column temperature of 20 °C and, a fluoxetine response was recorded. However, this fluoxetine peak was significantly broadened with extremely poor peak shape. As such, in effort to improve the peak shape, the oven temperature was increased to 50 °C and the injection was repeated. This condition resulted in only a slightly improved peak shape and Figure 4-7 shows that the fluoxetine peak profile was still significantly broadened. Therefore, this experiment demonstrates that the increased column temperature is aiding in the successful elution of fluoxetine. However, although even higher temperatures might potentially further improve the peak shape, they were not attempted since these high temperatures could readily result in the dissolution of silica and the destruction of the column and again, the venture seemed unpromising. The poor peak shape of fluoxetine eluting from the bare silica column could potentially be explained due to the ability of silica to hydrogen bond to the amine group of fluoxetine.[137] This ability to hydrogen bond could explain the strongly retained peaks. In this regard, it appears that, although the elution of fluoxetine from this column was recorded, bare silica is also not a viable stationary phase for the SWC elution of fluoxetine.

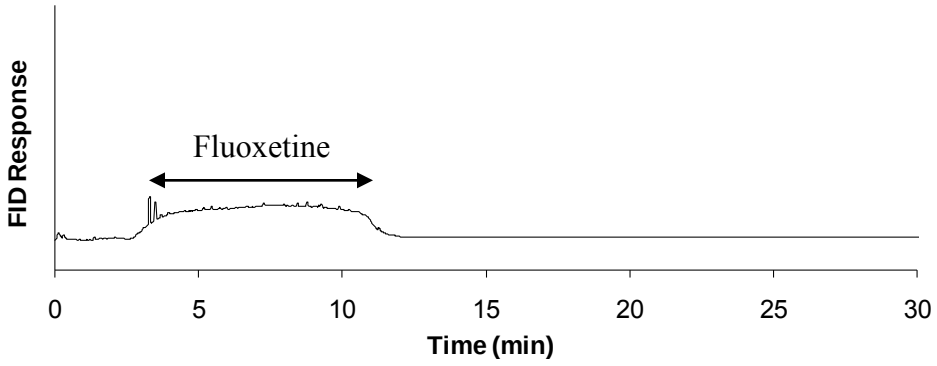


Figure 4-7: Conventional SWC elution of fluoxetine from a 50 mm × 250 µm I.D. (5 µm) bare silica packed capillary column at 50 °C. The water mobile phase flow rate is 10 µL/min.

4.4 CO_2 Modified SWC Method Development for Fluoxetine

Since the conventional SWC system had such a difficult time eluting fluoxetine from a variety of stationary phases, the CO_2 modified SWC system was next evaluated as a method for the analysis of fluoxetine. This blended mobile phase could potentially better elute fluoxetine, which was very highly retained on the columns employed in SWC, since CO_2 has the ability to decrease the net mobile phase polarity and significantly improve solvent strength in SWC. The PRP-1 column was employed in this mode of chromatography since it is less retentitive relative to the PBD column and it is unable to form dipole-dipole and hydrogen bonding interactions with fluoxetine which were possible in the cyano-bonded silica and the bare silica columns, respectively.

To begin, the conditions of 200 µL/min of water with 160 atm of CO_2 were first attempted in order to elute fluoxetine from the system. These conditions, with column temperatures of 20, 80, and 100 °C, resulted in no evidence of fluoxetine elution from the

column. However, immediately after only the fourth injection of fluoxetine onto the column (20.56 μg total injected), the system back pressure began to rise rapidly and the mobile phase flow rate dropped precipitously. Upon further investigation, the column itself appeared to be plugged. In order to better investigate the cause of the column blockage, solvent rinses were attempted to dissolve any highly retained compounds present on the column. Since flushes with acetonitrile failed to clear up the column blockage it seems unlikely that intact fluoxetine was simply stuck on the column in a similar fashion to what was observed in the SWC experiments. In addition, since stability studies indicate that fluoxetine is stable in water at 100 °C, it may also be unlikely that thermal degradation is responsible for the plugging of the column. One possible explanation for the column plugging is a reaction between the secondary amine functional group of fluoxetine with carbon dioxide, resulting in the formation of a carbamate. Such reactions can prove troublesome while analysing secondary amines in CO_2 based SFC.[138-139] Regardless of the cause of the column plugging, it appears that fluoxetine is incompatible with the CO_2 modified SWC system. As such, no CO_2 modified SWC method could be developed for the analysis of fluoxetine either.

4.5 Conclusions

Unfortunately, the efforts outlined in this chapter were not able to successfully lead to an analysis method for fluoxetine in either SWC or CO_2 modified SWC. In this regard, SWC elution of fluoxetine was not possible from PBD-clad zirconia or PRP-1 columns under the auspices of a maximum column temperature of 150 °C for 30 minutes. Further, cyano and silica columns yielded similar results at lower temperatures. In

addition, the CO_2 modified SWC system showed significant incompatibilities with fluoxetine which has resulted in irreparable damage to a separation column. However, one potential bright spot remains since the elution of fluoxetine was possible from a bare silica packed capillary column despite the poor peak shape. Although no successful method was developed for the SWC or CO_2 modified SWC analysis of fluoxetine, the results help to shed further light for future SWC method development studies on this molecule.

Chapter Five: **THE EFFECT OF ULTRASOUND IN CARBON DIOXIDE MODIFIED SUBCRITICAL WATER CHROMATOGRAPHY**

5.1 Introduction

Ultrasound describes sound frequencies above the scope of human hearing (i.e. above 20 kHz). Such frequencies in the 20 – 40 kHz range can induce very small void pockets within a fluid while passing through them. When this bubble collapses, a localized region of very high temperature and pressure is formed (5000 °C, 2000 atm).[140] Since these bubbles are very small and sparsely distributed throughout the fluid, this localized region of high temperature and pressure is quickly dissipated and has little effect on the bulk properties of the fluid. However, this interesting property is analytically relevant when one such cavity forms near a solid which is immersed in the fluid. Upon collapse of the bubble, the surface tension of the fluid is overcome and the fluid is better able to rush up into direct contact with the surface of the solid. For example, when this solid is a matrix particle in an analytical extraction, ultrasound has the potential to aid solvent penetration of the matrix which, in turn, speeds up the extraction process. Conversely, when the solid is a stationary phase particle in chromatography, the mass transfer of an analyte between the mobile phase and the stationary phase could potentially be sped up resulting in faster and more efficient separations.

Ultrasound has found the most analytical utility as an aid in extraction. The application of ultrasound has been shown successful in reducing solvent consumption, improving the extraction yield, lowering the operating temperature, and speeding up

extraction time in liquid-liquid extraction,[141] liquid-solid extraction,[142] and in supercritical fluid extraction.[143] While ultrasound has established itself as a useful analytical tool in sample preparation[144] and other analytical techniques,[145] it appears in the literature only sparsely as applied to analytical separations. Limited successful implementation of ultrasound in separations has been published for gel electrophoresis,[146] ion-exchange chromatography[147-148], and size-exclusion chromatography.[149] However, only one example in the literature was found applying ultrasound to HPLC. In this study, the applied ultrasound resulted in a small decrease in the analysis time and a minor improvement the resolution of chiral separations in HPLC.[150]

Although the effect of ultrasound acting directly on the separation mechanism in HPLC may be mild, ultrasound might prove useful in the CO_2 modified SWC method presented in Chapter Three. For example, under lower temperature conditions where CO_2 and water are relatively immiscible and provide unfavourable chromatographic conditions such as peak splitting, ultrasound could perhaps help promote better mixing of these mobile phase components and improve the chromatographic performance. Therefore, if so, the ultrasound-induced effect of better mixing could, in turn, potentially allow for operation at even lower temperatures in the CO_2 modified SWC system, which would further expand the separation conditions available to a wider range of analytes. This potential better mixing is supported by the effect that ultrasound can have on CO_2 and water media. For example, the ability of ultrasound to promote mixing between CO_2 and water has been demonstrated in the synthetic chemistry literature. In this regard, ultrasound has been employed to promote the formation of microemulsions between supercritical CO_2 and water which allow for the solvation of compounds that would not

normally be soluble in either pure fluid.[151] This emulsion of CO_2 and water has been successfully applied to increase the rate of reaction for chemical synthesis when compared to that measured in conventional solvents.[152-153]

Chapter Five investigates the possibility of applying ultrasound to the CO_2 modified SWC system presented earlier in Chapter Three, in order to promote better mobile phase mixing, and therefore better chromatographic performance, at even lower temperatures which could further expand the application of this mode of chromatography. The instrumental parameters described in section 2.3 are used in these experiments.

5.2 The Effect of Ultrasound on Conventional SWC, HPLC, and SFC

Initial efforts focused on establishing the effect that ultrasonic radiation had on separations with only a single (i.e. fully miscible) mobile phase present. Exploring ultrasound with a single, miscible phase was necessary since, if no effect was observed, these results could help support any observation of the ability of ultrasound to promote better mixing of the mobile phase components. As such, any direct effect on conventional separations was first probed.

Conventional SWC separations (i.e. with pure water and no CO_2 present in the mobile phase) were performed first, using a methanol and ethanol analyte test mixture. Ultrasonic energy could be provided by either the ultrasonic bath, the ultrasonic processor probe attached directly to the column, or both operating simultaneously, as seen in Figure 5-1. To begin, the ultrasonic bath was set to about 70 °C and comparative separations were performed with mobile phase flow rates of 1.0 mL/min, 0.5 mL/min, and 0.1 mL/min. In each case, only a minor decrease in analyte retention time was

observed upon the application of ultrasound. Curiously, however, the effect was observed only when ultrasound was provided by the bath and was not observed when using the ultrasonic processor attached to the column. Upon further investigation, it appeared that activating the ultrasound in the bath increased the bath temperature on the order of 1-2 °C. Accordingly, when the ultrasound was activated, a 1-2 atm decrease in column back pressure was also observed. The decrease in back pressure likely corresponds with the resultant viscosity drop due to the increase in mobile phase temperature. Therefore, it appears that the subtle decrease in retention time was not due to the influence of ultrasound but instead due to the subtle temperature increase also observed.

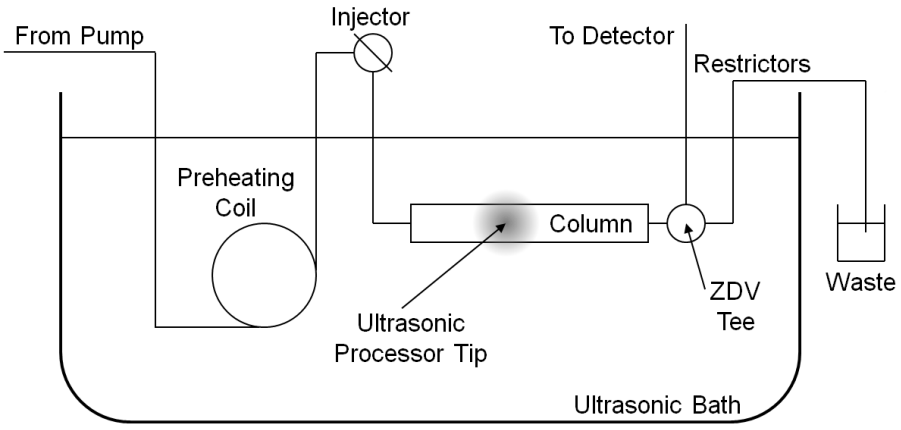


Figure 5-1: Application of ultrasound to the SWC system.

Next, a similar separation was performed with a conventional HPLC mobile phase of 60% methanol and 40% water with UV-Vis detection. A benzene and toluene mixture was separated on the PRP-1 column at a bath temperature of 30 °C. Separations were again performed with mobile phase flow rates of 1.0 mL/min, 0.5 mL/min, and 0.1

mL/min, with and without the application of ultrasound. Akin to the SWC separations, no effect was observed when ultrasound was provided by the ultrasonic processor in a similar fashion to Figure 5-1. However, in HPLC, no effect was also observed upon activation of the ultrasonic bath. Although a moderate increase in bath temperature and a corresponding decrease in system back pressure were recorded, no change in analyte retention was observed. Further, since the only observed effect of ultrasound can be explained by an increase in solution temperature, it can be concluded that ultrasound has no measurable effect on the separations observed in SWC and HPLC.

In addition to SWC and HPLC, the effect of ultrasound was also evaluated using SFC with a pure CO_2 mobile phase. In this mode, a 1-octanol test analyte was eluted at 60 °C with 160 atm of CO_2. The potential effect of ultrasound was assessed by comparing separations performed with no ultrasound applied, with ultrasound provided by the ultrasonic bath, with ultrasound applied through the processor attached to the inlet fitting of the column, and with ultrasound provided by both the bath and the processor, again in a similar fashion to Figure 5-1. In each case, no change in retention time, efficiency, or peak shape was observed in this system. In addition, although a small increase of temperature was observed when employing the ultrasonic bath, there was no resultant effect on the separation. Therefore, ultrasound also does not appear to have an effect on the separation when only CO_2 is employed as a mobile phase.

Overall, ultrasound showed no measurable effect of the separations performed in conventional SWC, HPLC, and SFC. The results of these experiments are summarized below in Table 5-1.

Table 5-1: Summary of the effect of applied ultrasound on bath temperature, column back pressure and analyte retention time for experiments performed in SWC, HPLC, and SFC.

Separation System	Source of Ultrasound	Change in Bath Temperature	Back Pressure Change	Effect on Retention Time
SWC	Bath	+ 2.7 %	- 5.2 %	Small decrease
SWC	Probe	No change	No change	None
SWC	Bath & Probe	+ 3.7 %	- 6.5 %	Small decrease
HPLC	Bath	+ 7.5 %	- 4.8 %	None
HPLC	Probe	No change	No change	None
HPLC	Bath & Probe	+ 7.8 %	- 5.0 %	None
SFC	Bath	+ 3.5 %	N/A*	None
SFC	Probe	No change	N/A*	None
SFC	Bath & Probe	+ 3.5 %	N/A*	None

* In SFC, the pump is operated in constant pressure mode.

5.3 Effect of Ultrasound on CO_2 modified SWC

Although the preceding experiments showed no obvious effect of ultrasound on separation systems employing conventional HPLC, SWC, or SFC mobile phases, there remained the potential that ultrasound could promote better mixing of water and CO_2 in the mixed mobile phase system. If better mixing of these components could be obtained, the dramatic peak splitting observed at lower temperatures (e.g. in Figure 3-2) could potentially be reduced or eliminated. In this regard, the effect of ultrasound was evaluated at a condition that would normally promote peak splitting (i.e. 200 μL/min of water and 160 atm of CO_2 with column temperatures less than 75 °C).

Initially, the effect of only the ultrasonic bath was evaluated using a 1-butanol test analyte. Separations were performed with and without ultrasound at column temperatures of approximately 40, 50, and 60 °C. In these experiments, no change in peak shape or retention time was observed upon activation of the ultrasonic bath. However, it should be noted that the trials performed at about 75 °C resulted in significantly reduced peak splitting regardless of the application of ultrasound. At this point, the temperature is sufficient for better mixing of the mobile phase components which results in the reduction of peak splitting. Although most of the splitting was reduced due to the increased temperature, no further change in peak shape was observed upon the activation of ultrasound. The results of these experiments are summarized in Table 5-2 below.

Table 5-2: Summary of the effect of ultrasound provided by the ultrasonic bath on 1-butanol retention time and peak shape in CO_2 modified SWC.

Bath Temperature (°C)	Ultrasound Applied?	Retention Time (min)	Change in Peak Shape?
40.6	No	1.55	No change
40.4	Yes	1.55	
50.3	No	1.50	No change
49.6	Yes	1.52	
63.7	No	1.77*	No change
63.8	Yes	1.82*	
74.2	No	1.54*	No change
74.2	Yes	1.55*	

* These trials were performed with different restrictors, hence the change in retention time.

Next, the effect of the ultrasonic processor was evaluated by attaching it to various regions of the separation system such as the mixing tee, the preheating coils, or the inlet fitting of the separation column as shown in Figure 5-2. In this regard, no appreciable change in the peak shape of 1-butanol was observed under any of these conditions at 60 °C. Similar experiments were next performed using both the ultrasonic processor and the ultrasonic bath simultaneously in order to provide even more ultrasonic

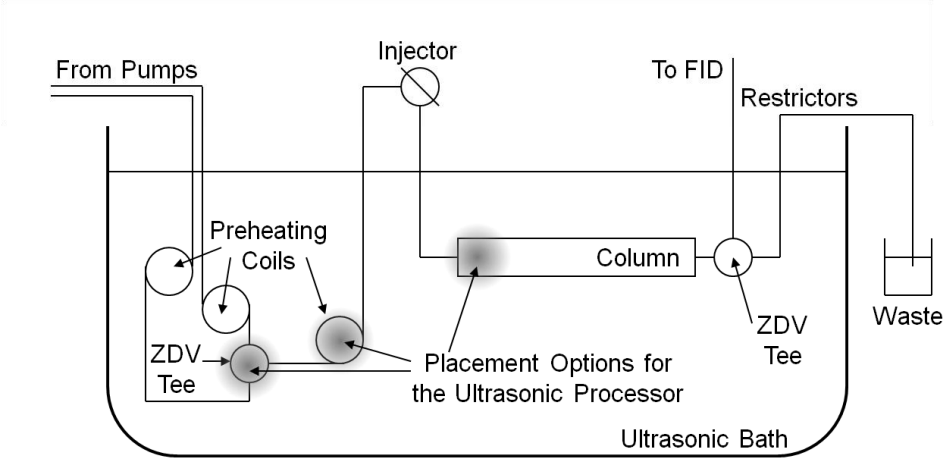


Figure 5-2: Application of ultrasound to the CO_2 modified SWC system.

energy to the system. In this case, an effect was observed at 60 °C when the ultrasonic processor probe was attached to the preheating coils after the mixing tee. A summary of these experiments is presented in Table 5-3 and an example of the resultant change in peak shape is shown in Figure 5-3.

Table 5-3: Summary of the effect of ultrasound provided by the ultrasonic bath and or the ultrasonic processor on 1-butanol peak shape in CO_2 modified SWC.

Application of the Processor	Ultrasonic Bath Applied?	Change in Peak Shape?
Column	No	No change
	Yes	No change
Mixing tee	No	No change
	Yes	No change
Preheating coil	No	No change
	Yes	Yes!

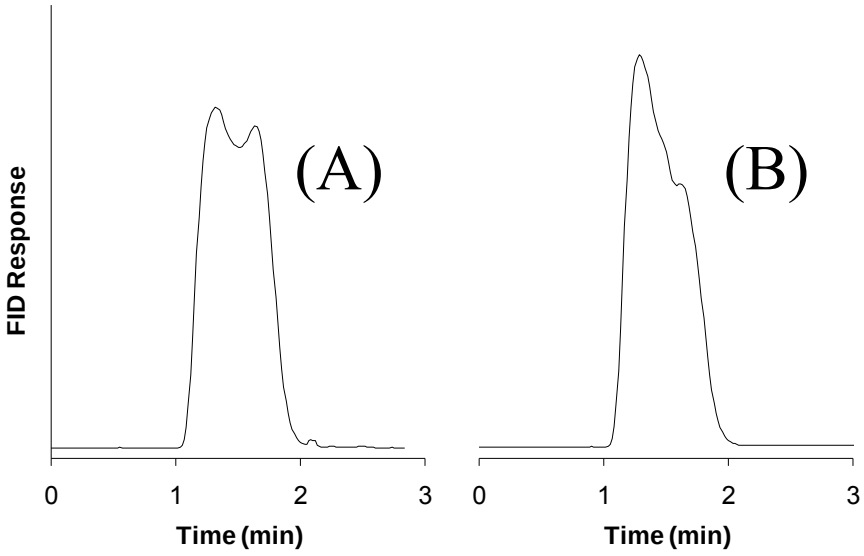


Figure 5-3: CO_2 / water elution of a 1-butanol test analyte (A) without and (B) with ultrasound applied to the system. In each case, the water flow rate is 200 µL/min, the CO_2 pressure is 160 atm, and the column temperature is very similar at (A) 63.8 °C and (B) 63.4 °C. 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column.

As observed in the Figure 5-3, the addition of ultrasonic energy to the preheating coils resulted in a minor change in the 1-butanol peak shape. The split peak profile observed in Figure 5-3A is consistent with other low temperature peak profiles and is indicative of poorly mixed mobile phase components. However, upon activation of ultrasound, the Figure 5-3B peak profile appears slightly less split which suggests that the mobile phase components are potentially better mixed. In addition, it appears that this effect was only observed when the ultrasound was applied to the preheating coils since no similar effect was observed when the probe was in contact with the mixing tee or the column itself. While the utmost care was taken to maintain a constant temperature between these separations, the bath temperature was about 0.5 °C lower when the ultrasound was applied in Figure 5-3B. This very small negative change in temperature should not have the ability to promote better peak shape in this system. Therefore, it appears as though ultrasound does have the potential to promote better mixing of the mobile phase components, albeit at a minor level.

5.4 Effect of Ultrasound on a Packed Capillary Column in SFC

One concern that arose while performing these experiments was regarding the ability of the thick stainless steel column walls to effectively conduct the ultrasonic energy to the interior of the column. Since no effect of ultrasound was observed while employing the 1/4 in. O.D. × 4.1 mm I.D. PRP-1 column in any of the conventional forms of chromatography such as HPLC, SWC, and SFC, additional experiments were performed to evaluate whether the stainless steel column tube may have damped the ultrasonic energy. For this purpose, a packed capillary column was prepared in-house

using a 15 cm length of 250 µm I.D. × 363 µm O.D. fused silica tubing. In this regard, comparative separations could be performed to determine if ultrasound can be successfully transmitted through the fused silica column wall and result in an effect on separations. SFC was selected as the separation medium since bare silica stationary phase particles were used to pack the column and SFC provides for good separations on this material.

Experiments were performed at about 60 °C with 100 atm of CO_2, using the ultrasonic processor as the only source of ultrasound in order to eliminate the possibility of temperature effects due to the ultrasonic bath. The ultrasonic processor probe was clamped onto the inlet union of the column in a similar fashion to the way it was connected to the stainless steel column, as shown in Figure 5-4. The elution of a 1-octanol test analyte with and without ultrasound is shown in Figure 5-5.

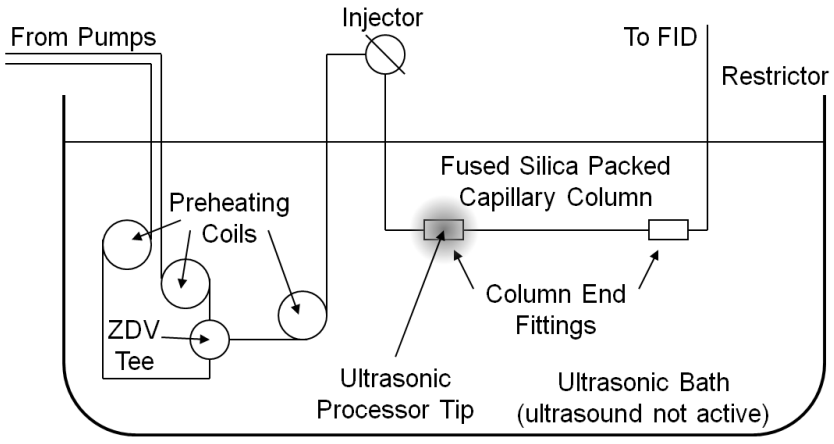


Figure 5-4: Application of ultrasound to packed capillary SFC system.

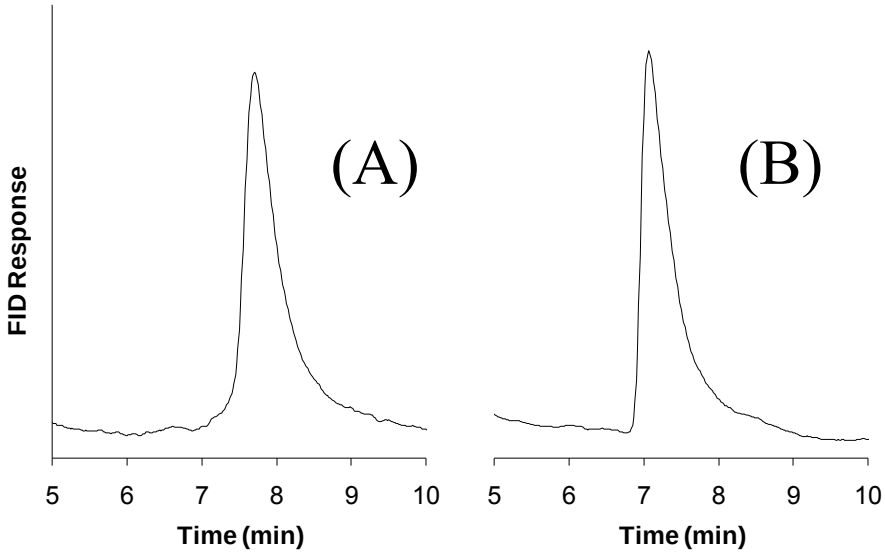


Figure 5-5: SFC elution of a 1-octanol test analyte (A) without and (B) with ultrasound applied to the system. In each case, the CO_2 pressure is 100 atm with a flow rate of 25 µL/min and the column temperature in both examples is 64.1 °C. 50 mm × 4.1 mm I.D. (5 µm) PRP-1 column.

Figure 5-5 demonstrates that, relative to the conventional stainless steel column results earlier, the ultrasonic processor seems to have a mild effect on the SFC elution of 1-octanol from a packed fused silica capillary column. For example, it appears that, upon activation of ultrasound to the system, the retention time decreases from 7.70 minutes to 7.07 minutes. In addition, a minor change in peak shape is evident as the peak width at half height is reduced from 29 seconds to 26 seconds. Correspondingly, the addition of ultrasound results in a mild increase in chromatographic efficiency as noted by the increase in the number of theoretical plates from 1409 to 1476. This change in efficiency

could be due to ultrasound speeding the mass transfer of the analyte between the mobile and stationary phases. Since all other operating conditions such as column temperature, CO_2 pressure, and CO_2 flow rate are held constant, it appears that ultrasound may have a minor effect on the separations performed within this system. However, the possibility of localized heating near the ultrasound connection also cannot be ruled out at this time. Therefore, it may be suggested that the stainless steel column housing employed in the earlier conventional separations perhaps damps the ultrasonic energy, thereby preventing it from reaching the column interior and affecting separations. Conversely, since the packed capillary housing wall is significantly thinner and made of a different material, perhaps it is better able to conduct the ultrasound directly to the stationary phase. However, further experimentation is required to confirm this.

5.5 Conclusions

These studies show that ultrasound has the potential ability to promote better mixing of the CO_2 and water components of the mobile phase in the CO_2 modified subcritical water system. This is demonstrated by the noticeable improvement in the peak shape and decrease in the peak splitting observed for a test analyte. However, it is likely that the source of ultrasound employed is not strong enough to promote complete mixing of these mobile phase components under the conditions used. In this regard, previous research has found that a minimum of 0.5 W/cm^3 of ultrasonic energy was required for effective emulsification of water and CO_2.[151-153] Conversely, the ultrasonic bath employed in these experiments was only able to provide about 0.024 W/cm^3 of ultrasonic energy. Therefore, it is likely that a stronger source of ultrasound could promote much

better mixing of the mobile phase components. This, in turn, would possibly enable separations to be performed at lower temperatures without the appearance of peak splitting, thereby expanding the range of applicable analytes to this mode of chromatography.

The results of these experiments also seem to indicate that ultrasound has the potential to offer beneficial improvements to separations performed in capillary columns, more so than in conventional stainless steel columns. The changes in retention time and improvement in efficiency are consistent with previous observations noted in ion chromatography.[148] The authors of this study also concluded that the 200 W ultrasonic bath employed was too weak of an ultrasound source for dramatic changes to the separation. This observation correlates with our findings in that the ultrasound source may again be too weak to penetrate a conventional HPLC steel column since some effect was noticed with a smaller column. However, it appears that the experiments performed in this chapter show some future promise for ultrasound in chromatographic separations, even though the limiting factor of this application is the strength of the ultrasound source. As such, the analytical utility of ultrasound may improve accordingly if more powerful sources of ultrasound are developed. Regardless, since the results observed were moderate and a more powerful source was not available, this was not further pursued in this work.

Chapter Six: **CHROMATOGRAPHY USING A WATER STATIONARY PHASE AND A CARBON DIOXIDE MOBILE PHASE**

6.1 Introduction

Water has long been employed in analytical separations as a constituent of the mobile phase. For example, water has been used for years as a primary mobile phase component in HPLC, often in combination with various organic solvents and/or additives. In addition, as discussed above, water has found a niche role as a mobile phase in SWC. Further afield, water has also been added in small quantities to humidify the mobile phase in packed and capillary column GC[154-155] and SFC[156-157].

Comparatively, the incorporation of water as a stationary phase component in chromatography has been explored and utilized to a much lesser extent. For example, there have been a few reports of water forming a coating on solid support particles in the early development of GC.[158-161] Although water's use as a GC stationary phase coating was initially limited in its analytical utility due to its polar nature and in-situ volatility, it did prove very useful in physical chemistry applications to quantify various interfacial[162-163] and solution[164-165] properties of molecules. Subsequently, aqueous solutions containing various ions have been used in analytical GC as coatings on solid particles in order to alter analyte selectivity.[166-167] Beyond this, there have also been some isolated reports of the use of water as an HPLC stationary phase, either coated on a solid particle[168] or as a pure, solid, water-ice phase itself.[169]

As well, in relation to this, liquid water has also been used to form a pseudo-stationary phase in the absence of any solid support particles in a number of alternative

separation techniques such as counter-current chromatography (CCC)[170], co-current chromatography[171], and radial tube distribution chromatography.[172]

As described in Chapter Three, the addition of CO_2 to the water mobile phase of SWC significantly increases the mobile phase elution strength while reducing the necessity for high temperatures. While working with this system under certain conditions, it was observed that separations would sometimes occur even without a conventional chromatography column present (i.e. with the packed column removed and only stainless steel transfer lines present). Upon further careful optimization of the separation conditions, a means of reproducibly depositing water (saturated with CO_2) on the inside walls of an uncoated capillary and performing separations using a CO_2 (saturated with water) mobile phase has been established.

While other related separation systems employing immiscible gas and/or liquid phases have been reported,[173-174] some applications specifically utilizing supercritical CO_2 have been described. For example, CCC using a supercritical CO_2 mobile phase was employed to separate acetophenone and benzophenone in 3 hours when incorporating a rotating coil operating at 435 rpm.[175] More recently, however, methanol/supercritical CO_2 systems have been explored by Parcher, *et.al.*, which employ a slowly moving, pseudo-stationary methanol phase formed along the inner wall of an uncoated capillary.[176-177] In this instance, the separation of several light n-alkanes was clearly and successfully demonstrated. Conversely, a system as described above, which operates with a purely aqueous stationary phase and a CO_2 mobile phase in capillary separations has not been reported.

In contrast to the CO_2 and methanol system described above, the CO_2 and water separation system described herein offers interesting and advantageous properties that stem from the very limited miscibility of water and CO_2. For instance, they allow for relatively aggressive mobile phase temperatures and pressures to be used to improve analyte solubility in CO_2 without degrading or collapsing the water phase. Also, under these conditions, the water phase appears to be rendered completely immobile inside the capillary which provides for a truly stationary phase. Further, since both water and CO_2 are unresponsive in a FID, this inexpensive and universal detector can be readily used in this mode of chromatography. Also of note, in contrast to CCC, which utilises mechanical rotation to operate efficiently, the water phase in this system is held stationary only by attraction to the stainless steel wall and does not require centrifugal forces to operate. Finally, this system offers good flexibility in method development through the control of operating parameters such as column temperature, CO_2 flow rate and CO_2 pressure.

Chapter Six describes the general characteristics and operating parameters of this separation system as performed in a capillary tube using water (saturated with CO_2) as a stationary phase and CO_2 (saturated with water) as a mobile phase (simply referred to herein as the water stationary phase and the CO_2 mobile phase respectively). The properties of this novel separation method will be illustrated using several different test analytes and its employment in various analytical applications will also be presented and discussed. The instrumental parameters described in section 2.4 are used in these experiments.

6.2 General Operating Characteristics

In general, analyte separations in this system appear to be classically partition-oriented and based upon a solute's relative solubility in either phase. Further, due to water's polar nature, separations tend to follow a normal phase pattern with the least polar analytes eluting first. Given these observations, initial efforts focused on exploring and optimizing the operating parameters of this separation system. As such, experiments were performed in order to better understand the effect that operating parameters such as column material, applied CO_2 pressure, column temperature, mobile phase flow rate, and column dimensions have on analyte separations.

6.2.1 Column materials

First, separations of a model n-alcohol mixture were performed to compare capillaries made from different materials. Those compared were stainless steel, fused silica and polyether ether ketone (PEEK), which were all of equal dimensions and operated under the same conditions. Figure 6-1 shows the typical results of these separations and demonstrates that the column material indeed plays an important role in establishing the water stationary phase. For example, the stainless steel column (Figure 6-1A) performed as expected, whereas no separation at all was observed in the PEEK column (Figure 6-1C). Conversely, the fused silica column (Figure 6-1B) appeared to have intermediate performance between these two. In particular, although the heavier alcohols appear to be more retained in the fused silica capillary than the stainless steel capillary, the lighter alcohols appear to be less retained. Analyte resolution is also clearly reduced in the fused silica capillary. Therefore, the material that the capillary is made of

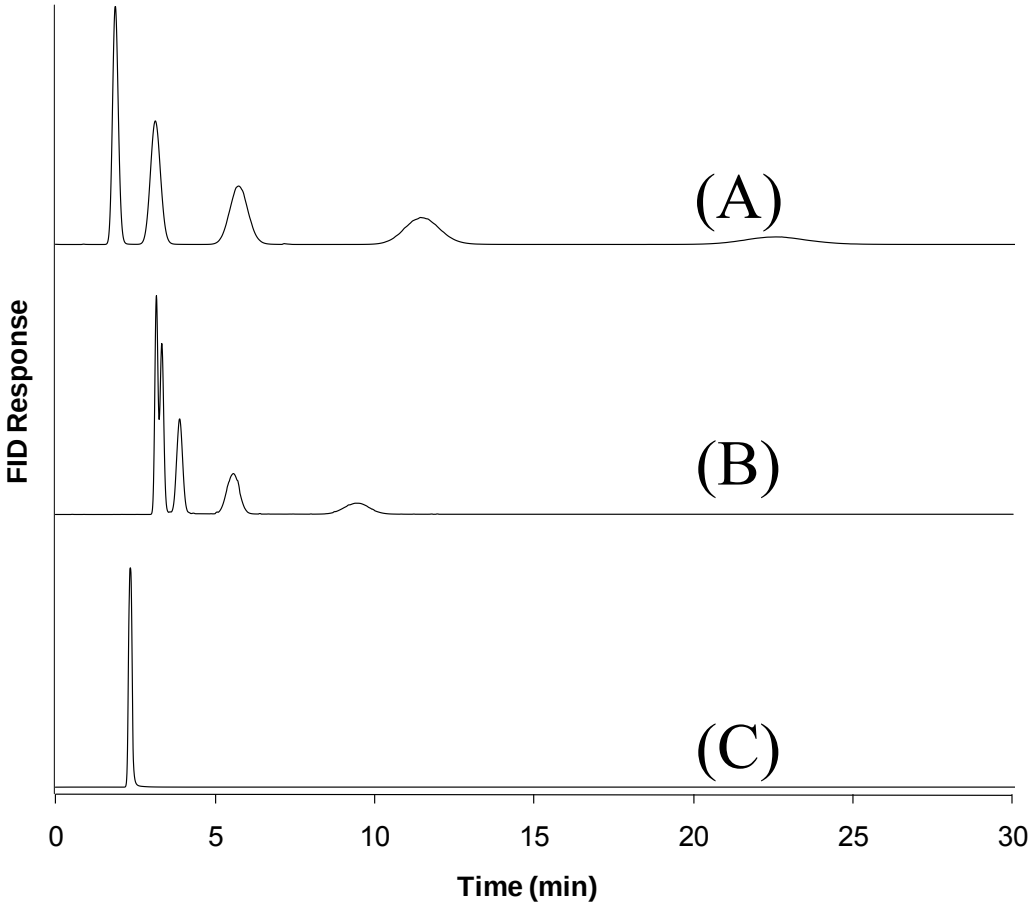


Figure 6-1: C_1-C_5 n-alcohol injections on different column materials at 20 °C with 80 atm of CO_2 (~200 µL/min) and 0 µL/min of water. The materials are (A) Stainless steel, (B) Fused silica, and (C) PEEK. All columns are 10 m × 250 µm I.D. Peak heights are normalized for clarity. Elution order: pentanol, butanol, propanol, ethanol, methanol.

and its relative capacity to allow water to spread over its surface as a stationary phase can have a large impact on analyte separation. This is further supported by surface-water contact angle measurements that indicate that PEEK is the most hydrophobic of these, followed by quartz and stainless steel.[178-180] However, the surface-water contact angles of

stainless steel and quartz are very similar and may not explain the difference of analyte retentivity on these columns. Perhaps then, an alternate explanation for the increased retention on the stainless steel column over the fused silica column could result from the differences in surface properties between these two tubes. Due to the manufacturing processes involved, fused silica has a relatively very smooth, uniform surface which is made up of the same material throughout. Conversely, stainless steel has considerably more surface roughness and is composed of a mixture of various metal components of the alloy. Therefore, perhaps the increased surface area and varying surface properties of the stainless steel column allow for more water to coat the inside, thereby increasing the retention of analytes on a column made of this material as compared to fused silica. It is further worth noting that fused silica separations had to be performed at room temperature since the column is readily destroyed after contact with hot water for any prolonged period of time.

6.2.2 CO_2 pressure

Next, we investigated the effect of applied CO_2 pressure using the alcohol mixture over a range of differing conditions. As expected, an increase in pressure resulted in a decrease in retention time. For example, as seen in Figure 6-2, an increase from 80 atm to 120 atm at 50 °C with a constant CO_2 flow rate resulted in a 45% reduction in the alcohol elution time due to greater analyte solubility in the denser mobile phase. While this general trend applied to all of the analytes investigated, it should be noted that an interesting observation arose while probing the effects of CO_2 pressure for a phenol and methanol test mixture. Specifically, as depicted in Figure 6-3, the elution order of these

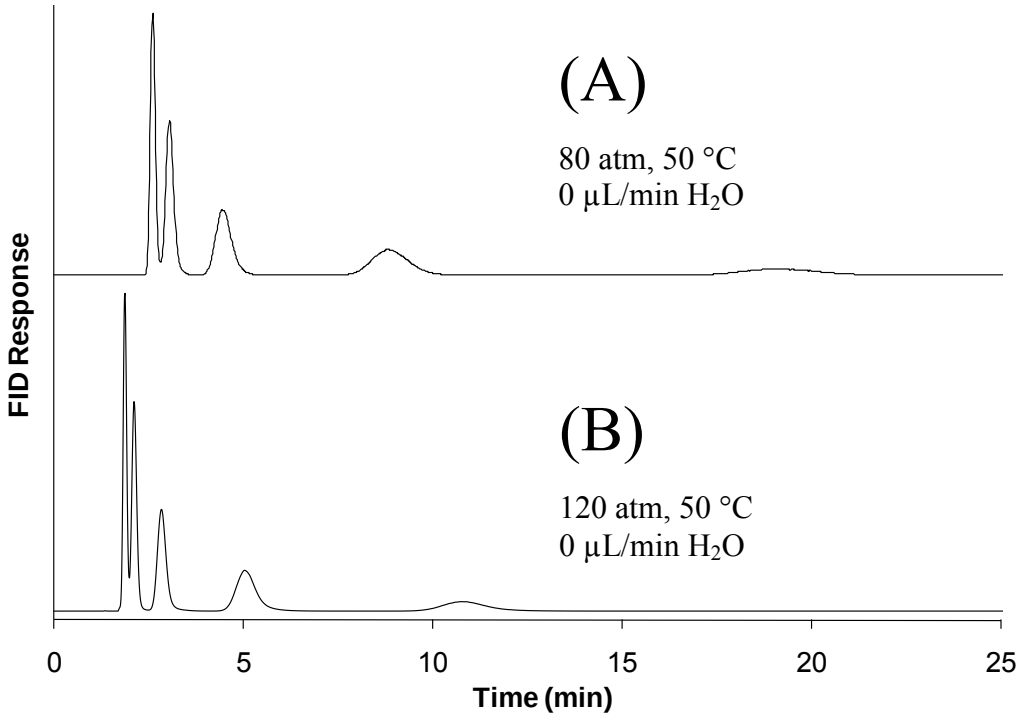


Figure 6-2: Separations of a model C_1-C_5 n-alcohol mixture at different pressures / densities. A constant CO_2 flow rate of 200 µL/min was employed. Elution order is the same as in Figure 6-1. 10 m × 250 µm I.D. stainless steel column.

analytes reversed after an increase in pressure from 80 to 130 atm under constant temperature and CO_2 flow rate. Although the reason for this is not entirely clear, it is believed that this change in elution order may be due to the nearly two-fold increase in the CO_2 solubility of phenol when raising from 80 to 130 atm.[181] As such, this may result in a more significant reduction in the phenol retention time relative to methanol, which is typically readily soluble in CO_2. Upon further investigation, this peak reversal trend was also observed to proceed in a linear fashion over a range of pressures as seen in Figure 6-4. At low pressures, the methanol peak elutes first, but as the pressure approaches 100

atm the two species co-elute. Then, above 100 atm, the phenol peak begins to elute first and continues this trend becoming more highly resolved from the methanol peak. Therefore, in certain cases, altering the CO_2 pressure may provide another route to control analyte selectivity and partitioning.

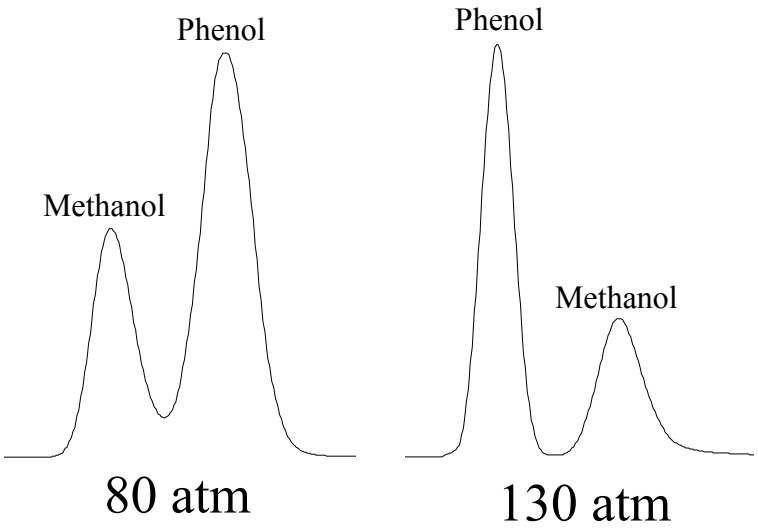


Figure 6-3: Phenol and methanol elution at different CO_2 pressures and a constant CO_2 flow rate of ~200 µL/min with 1 µL/min of water at 100 °C. Peak heights are normalized for clarity. 10 m × 250 µm I.D. stainless steel column.

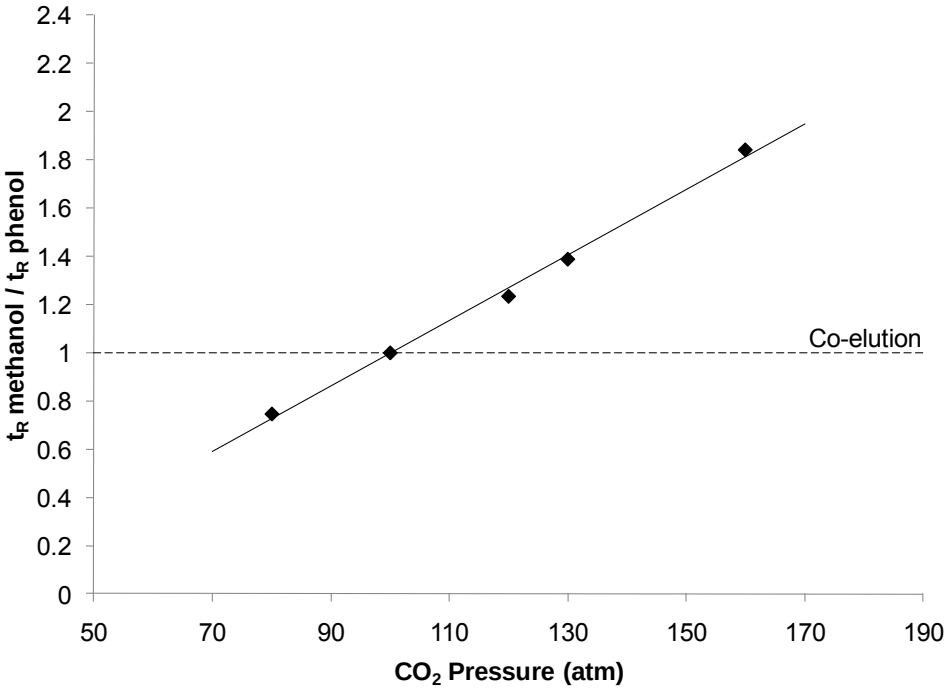


Figure 6-4: Relationship between CO_2 pressure and the methanol / phenol elution order. Each run was maintained at a CO_2 flow rate of about 200 µL/min with 1 µL/min of water at 100 °C. 10 m × 250 µm I.D. stainless steel column.

6.2.3 Column temperature

Next, as shown in Figure 6-5, we looked into the effect that column temperature had on separation. It was found that increasing the temperature from 20 to 100 °C at constant CO_2 pressure resulted in about a 4-fold reduction in the total elution time of the n-alcohol mixture. Clearly temperature also has a profound effect on retention in this system since it has the potential to affect key separation variables in this regard such as the polarity of the water phase,[28] its interaction with CO_2,[114,182] and solute vapour pressure. Therefore, both pressure and temperature provide influential parameters for optimizing separations when using this method.

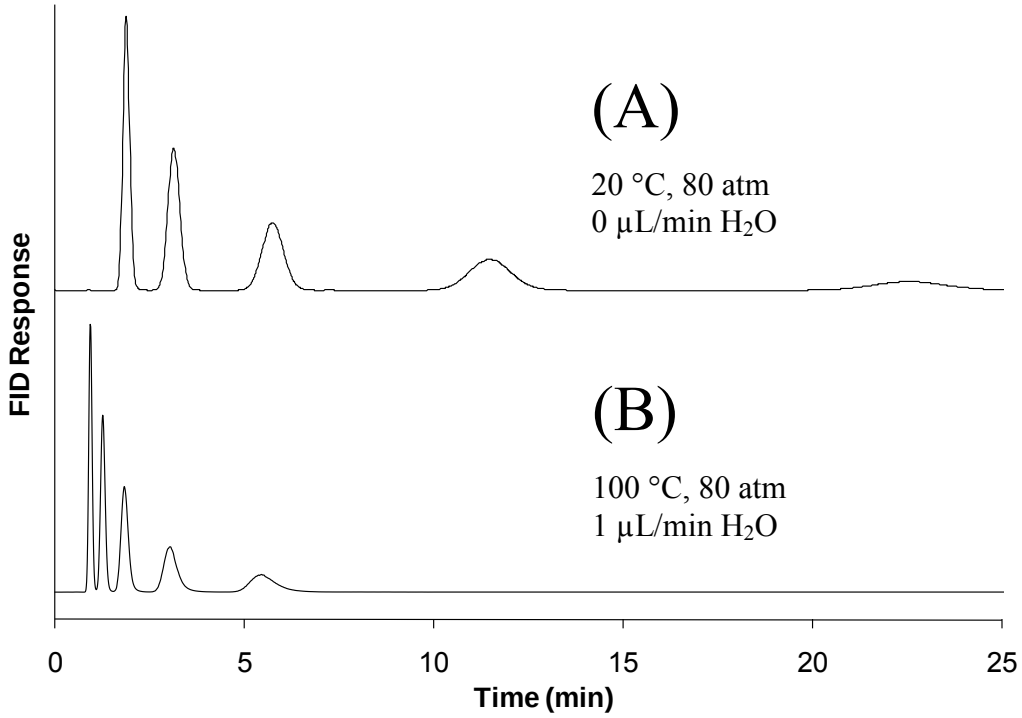


Figure 6-5: Separations of the C_1-C_5 n-alcohol mixture at different temperatures using a constant CO_2 pressure of 80 atm (200 µL/min). Elution order is the same as in Figure 6-1. 10 m × 250 µm I.D. stainless steel column.

6.2.4 CO_2 flow rate

Also of interest was the effect that the CO_2 mobile phase flow rate had on separation. In general, it was found that while holding temperature and CO_2 pressure constant, typical CO_2 flow rates ranging from about 120 to 400 µL/min did not appear to greatly alter separation efficiency. (Note: in order to do this, the restrictors were changed. See experimental.) However, as expected, some erosion in efficiency was seen at larger flow rates. Of note, CO_2 flow rates above about 450 µL/min resulted in a gradual collapse

of the system's separation ability due to the evaporation of the water stationary phase. However, in such instances we have found that simply increasing the water flow rate can often offset this effect and allow for such faster CO_2 flow rates if required.

6.2.5 Column dimensions

Additionally, column dimensions were investigated for their effect on separations. As expected, changes in column length had a direct effect on efficiency and retention. For example, a reduction in column length from 10 m to 5 m resulted in about half the separation efficiency and retention time for test analytes. It was also found that decreasing the column I.D. would often provide relatively faster separations, mostly owing to the inherent increase in linear velocity. However, the corresponding impact on separation efficiency was also apparent. For instance, using a 125 µm I.D. column typically resulted in faster separations and notably improved resolution overall. Table 6-1 illustrates this for a highly retained alcohol, methanol, and demonstrates how its retention and resolution from a neighbouring ethanol peak are impacted by changes in column dimensions.

Further, even smaller I.D. tubing was also attempted in this method. Due to its availability in small diameters, uncoated and undeactivated fused silica tubing of 100 and 50 µm I.D. was also evaluated. As such, the 100 µm tubing was able to support separations in a similar fashion to the 125 µm stainless steel column with differences in retention consistent with the observations made previously (section 6.2.1). However, separations proved troublesome with the 50 µm fused silica tubing. In this case, the significant difference in the viscosity of CO_2[183] and water[184] prevented the optimization of the CO_2 flow rate. In this regard, when a restrictor was selected to provide an optimum

CO_2 flow rate, the time required for the establishment of the stationary phase was unreasonably long. Conversely, if a restrictor was selected to allow for the column to equilibrate in a timely fashion, the resultant CO_2 flow rate would be unreasonably high and cause the rapid elution of analytes with no separation. As such, separations were only achieved with 100 µm I.D. or greater columns.

Table 6-1: Effect of column dimension on retention time and separation efficiency at 100 °C with 80 atm of CO_2 (~160 µL/min) and 1 µL/min of water.

Column Length (m)	Column I.D. (µm)	Methanol Retention Time (min)	Ethanol / Methanol Resolution
10	1000	17.52	1.94
5	1000	11.59	0.95
10	500	9.76	1.49
10	250	5.44	2.40
10	125	3.80	3.48

In addition to open tubular columns, separations were also attempted on a column filled with stainless steel particles. In this regard, a 15 cm length of 1 mm I.D. stainless steel tube was gravity packed with -325 mesh (~44 µm) stainless steel powder. The water phase was established and separations were attempted. Unfortunately, this column did not provide any retention of compounds under a range of conditions. Therefore, at this time, it appears as though a packed column is unable to provide for separations in a similar fashion to the open tubular column.

6.3 Water Stationary Phase Properties

6.3.1 Maintenance of the phase

Efforts to study the nature of the water stationary phase were performed next. Similar to early uses of water in GC,[159-162,164-165] a continuous addition of water to the mobile phase was required here to maintain the water stationary phase at temperatures near or above 100 °C. However, no water addition was required at lower temperatures. These findings indicate that water addition in this case effectively humidifies the column interior in order to minimize evaporation of the water stationary phase. Further, the depletion of this phase could be indirectly observed from the deterioration of the chromatographic performance. In this regard, water flow rates that were too high often resulted in excessive detector noise, whereas those that were lower than optimum sometimes resulted in the gradual depletion of the water phase and erosion of the separation efficiency.

In order to support these observations, an experiment was performed with repeated injections of an n-alcohol test mixture at 10 minute intervals as demonstrated in Figure 6-6. As expected, the separation steadily repeats until the water flow rate is stopped. The separation then gradually degrades over the next 60 minutes, after which the water phase has depleted to the point where separation is no longer possible and all analytes co-elute. Thus proper humidification can be important in order to maintain the water stationary phase. Interestingly, when the small water flow required for humidification is reactivated, the stationary water phase will slowly re-establish itself, reflecting the dynamic nature of the system. For example, when the 1 μL/min water flow rate was restored after the phase collapsed in Figure 6-6, about 3 hours were required to

re-establish the water phase. This required time would be reduced by increasing the water flow rate.

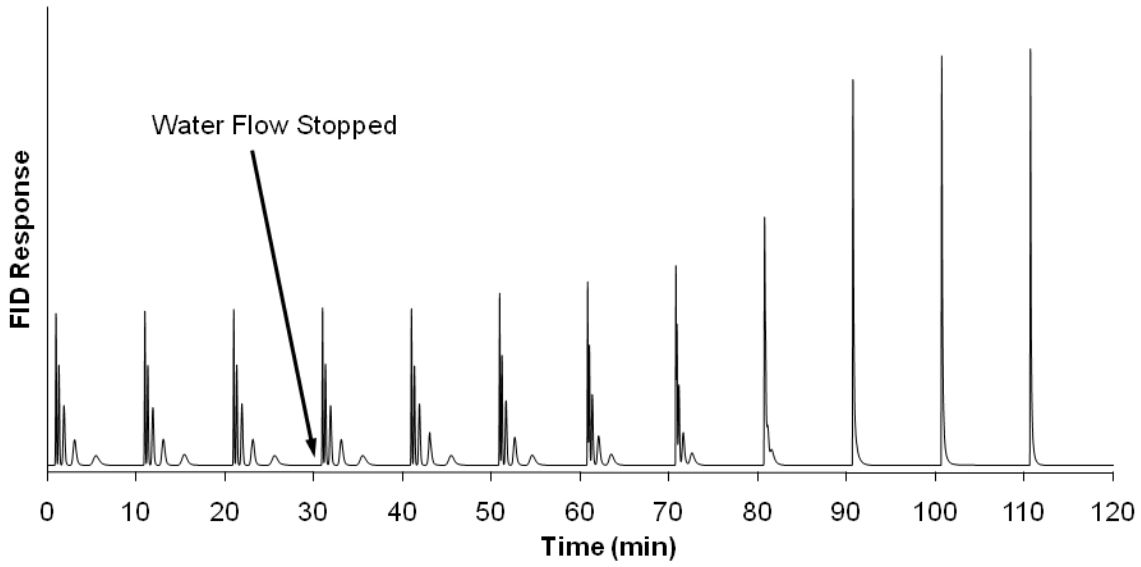


Figure 6-6: Repeated C_1-C_5 n-alcohol injections every 10 minutes at 100 °C with 80 atm of CO_2 (~200 µL/min) and 1 µL/min of water. The water flow rate is stopped at 30 minutes. Elution order is the same as in Figure 6-1. 10 m × 250 µm I.D. stainless steel column.

Of course, different column temperatures require different flow rates of water to maintain the stationary phase. For example, only 1 µL/min of water flow was required at 100 °C, whereas 30 µL/min was required at a column temperature of 250 °C. However, in contrast to this, at ambient temperatures no humidification was required and the water stationary phase remained intact, effective and unchanged when observed over a period of more than 18 hours. Therefore, while humidification is important, it is only necessary

at higher temperatures. Also, analogous to this, as CO_2 flow rates increase to larger values, greater water flow rates are again often required for proper humidification in order to prevent the stationary phase from evaporating.

6.3.2 Reproducibility

Another feature of this CO_2 / water system that can be noted Figure 6-7 is that the separations are readily reproducible. Although changes to the stationary phase have a significant effect on analyte retention, once the water stationary phase is established and maintained, it provides stable separations over long periods of time. For instance, typical repeated injections of 1-propanol produced retention times and plate heights with respective %RSD values of 1.0% and 7.6% (n=5). Further, from run-to-run where the stationary phase was intentionally eliminated and then re-established between trials,

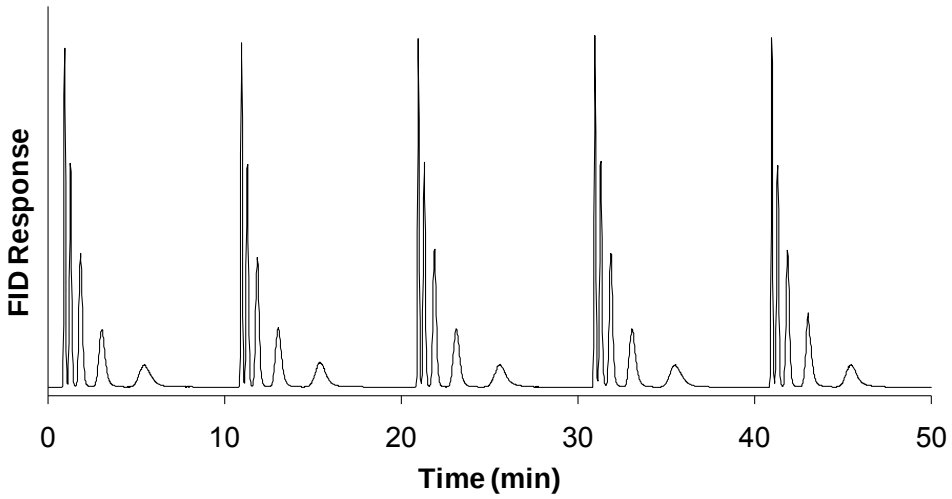


Figure 6-7: Repeated C_1-C_5 n-alcohol injections every 10 minutes at 100 °C with 80 atm of CO_2 (~200 µL/min) and 1 µL/min of water. Elution order is the same as in Figure 6-1. 10 m × 250 µm I.D. stainless steel column.

1-propanol injections yielded retention times and plate heights with respective %RSD values of 3.9% and 7.5% (n=8). Thus, for replicate injections and day-to-day operation, the separations reproduce very well.

6.3.3 Phase volume

Finally, in order to estimate the volume and thickness of the water stationary phase, it was repeatedly collected and weighed. This was accomplished by first establishing the water stationary phase, then stopping the water flow and purging the column with pure, dry CO_2 (100 °C, 80 atm) for two hours while carefully collecting the contents of the column in a covered vessel (open to atmosphere) cooled with dry ice. The mass of water collected was then measured and its volume was determined. These efforts resulted in a water stationary phase volume of 33.2 ± 1.0 μL (n=3). If this volume were to form a continuous, uniform coating on the inner wall of the column, then it would translate into a water stationary phase with a thickness of about 4.31 ± 0.13 μm. However, it is currently unknown if the water phase forms a uniform layer throughout the interior of the capillary or if it forms a series of discrete droplets. Either way, the consistent volume determined indicates that the surface coverage of the capillary inner wall is quite reproducibly established from run-to-run.

6.4 Mobile Phase Alternative

In order to evaluate the ability of an alternative to the CO_2 mobile phase in this mode of chromatography, separations were attempted with a nitrogen carrier gas. In this regard, the water phase was established as normal in a 10 m × 250 μm I.D. stainless steel

column and allowed to equilibrate with 120 psi of nitrogen. A separation of the n-alcohol mixture was performed and is shown in Figure 6-8. As seen in this figure, the peaks are broadened and more highly retained relative to a CO_2 mobile phase, but the elution order remains the same. Although successful in separating the relatively volatile n-alcohol mixture, the nitrogen carrier gas could not elute a less volatile menthol test analyte which was readily eluted with the CO_2 mobile phase. Therefore, since the nitrogen carrier gas lacks the solvating ability of the CO_2 mobile phase, the analytes amenable in this mode appear to be those that are more volatile, consistent with conventional GC. Further investigation with the nitrogen carrier gas was not pursued, but this GC mode appears promising.

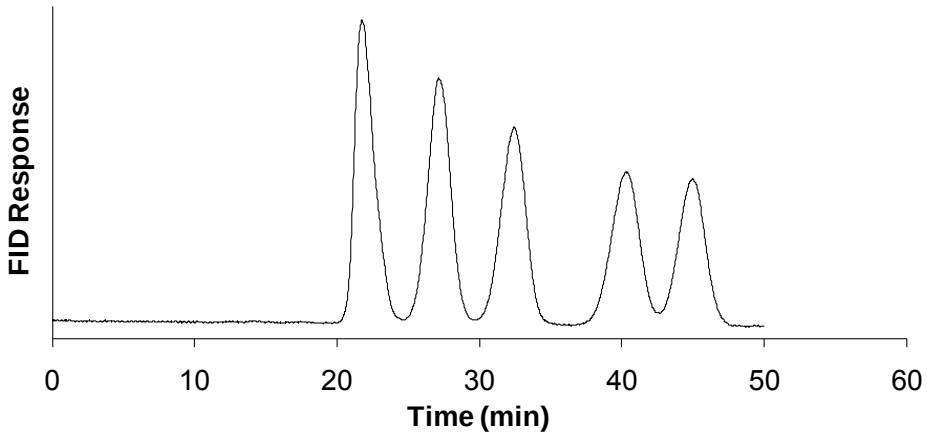


Figure 6-8: Separation of the C_1-C_5 n-alcohol mixture at 24 °C with a nitrogen carrier gas at a pressure of 120 psi (24 mL/min). Elution order is the same as in Figure 6-1. 10 m × 250 µm I.D. stainless steel column.

6.5 Separation Mechanism

6.5.1 Stainless steel – analyte interaction

Experiments were performed to determine if any of the separation observed was due to analyte interaction with the surface of the stainless steel capillary. The alcohol test mixture was injected into the stainless steel capillary without any water present and only CO_2 mobile phase flowing through the column. As shown in Figure 6-9A, under these conditions, no separation was observed and all analytes co-eluted as one peak. Similarly, when only water was flowing through the stainless steel capillary and no CO_2 was present, separation of the alcohol mixture was again not observed and all peaks co-eluted, as seen in Figure 6-9B. Thus, these findings indicate that there is no analyte retention or separation arising from interactions with the stainless steel capillary inner surface and that both CO_2 and water must be present in order to separate compounds in this system.

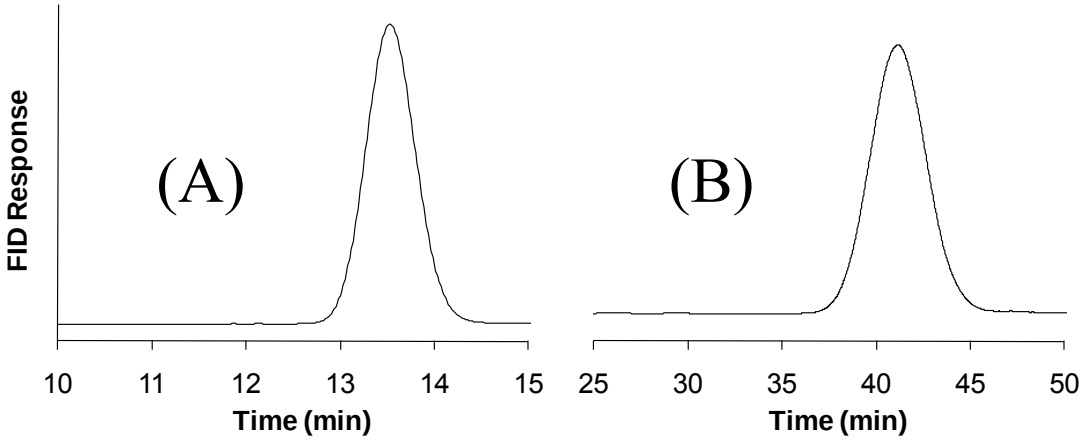


Figure 6-9: Elution of the n-alcohol test mixture from the 10 m × 250 µm I.D. column at 100 °C with (A) no water present and 80 atm (400 µL/min) of CO_2, and (B) no CO_2 present and 200 µL/min of water. The n-alcohols are co-eluting.

6.5.2 Analyte character

In order to better understand the effect that analyte character has on separations, two analyte classes of extremely different nature were examined. The first had very limited water solubility and high CO_2 solubility while the second had very high water solubility and limited CO_2 solubility. Such parameters were selected to further probe how analytes interact with the mobile and stationary phases and separate in this system. Using a standard set of conditions of 100 °C with 1 μL/min of water flow and 80 atm of CO_2 (200 μL/min), the first analyte condition was examined. For this, a mixture of n-alkanes in carbon disulfide was selected as an example of compounds with high CO_2 solubility and very limited water solubility. Upon injection, no separation of these analytes was observed and they rapidly co-eluted as a single, unretained peak, as shown in Figure 6-10A. Next, citric acid was selected as a compound with large water solubility and limited CO_2 solubility. As observed from Figure 6-10B, upon injection of this compound, no peak elution was detected even after several hours of operation. Further, when this experiment was repeated with a 1 m column using the same conditions, again no peak elution was observed after several hours of operation. In fact, not even a slight rise or perturbation in the baseline was noted during this long period.

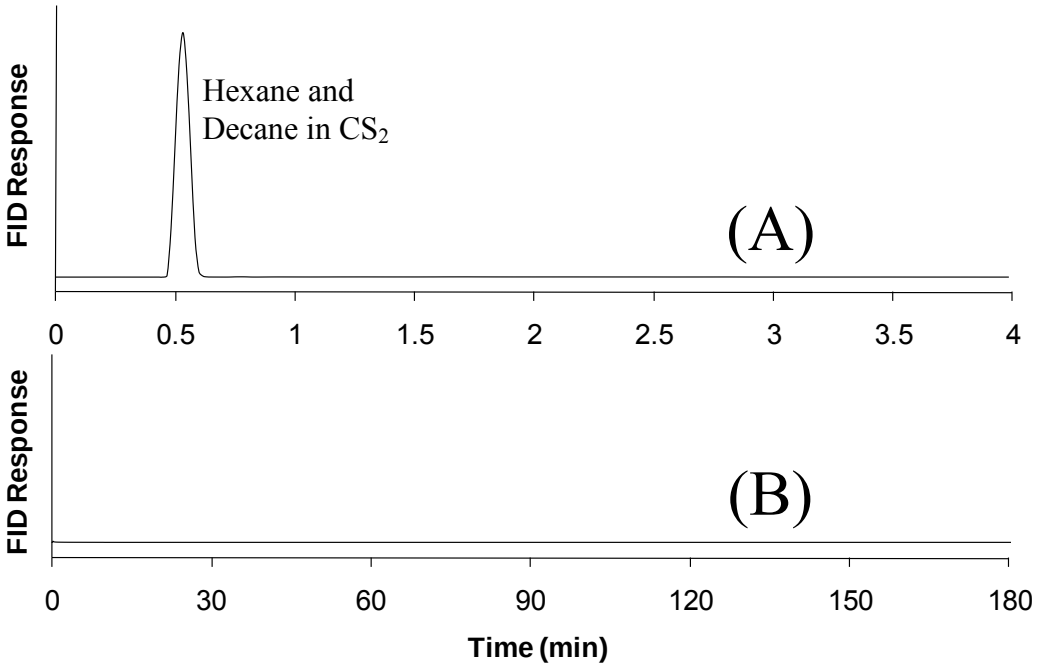


Figure 6-10: Injection of (A) n-alkanes in CS_2 and (B) citric acid in water onto the system at 100 °C with 80 atm of CO_2 (~200 µL/min) and 1 µL/min of water. 10 m × 250 µm I.D. stainless steel column.

These findings strongly indicate that even though a slight flow of water is used under these conditions to prevent evaporation of the wall coating (i.e. since the stationary phase is likely in dynamic equilibrium with the mobile phase[176]), the water phase is truly stationary and not moving along the inner wall since no trace of response from the injected citric acid could ever be detected. If the addition of water was to cause movement of the water phase, we would expect to see citric acid elute from a 10 m column after about 33 minutes (i.e. 1 µL/min of water added and 33 µL of water phase volume) or after only about 3.3 minutes from a 1 m column (i.e. 3.3 µL water phase volume). However, since we did not observe citric acid under either of these conditions

after several hours of operation, it stands to reason that the water flow rate is not causing movement of the stationary phase but rather providing humidification to prevent evaporation. This characteristic is in contrast to other biphasic CO_2 systems that are based upon a pseudo-stationary wall coating moving at a slower rate than the CO_2 mobile phase, which dictates that analytes can elute within a specified time frame.[173,176-177]

The investigations above also confirm that a compound appears to require some reasonable solubility in both the water and CO_2 phases in order to be retained and separated in this system. However, even moderate to low solubility in water appears to be sufficient to promote analyte retention in this system. For example, tocopherol has a very limited solubility in water,[185] but can still be retained in this system when appropriate conditions are chosen. Figure 6-11A demonstrates a tocopherol peak eluting in just under five minutes. Thus, despite its long, hydrophobic side chain, the sole hydroxyl group and ether linkage on this molecule appear to be effective in facilitating its retention in the system. Even more surprisingly, methylated tocopherol is also retained in this system under the same conditions. Figure 6-11B also demonstrates a methylated tocopherol peak also eluting in under five minutes. In this case, the sole hydroxyl group of tocopherol is now replaced with an ether linkage. In this regard, it appears that only the presence of ether linkages is sufficient for retention in this system. Therefore, this feature of the system could be advantageous for analyzing such large molecules in their native state, since analogous GC methods use aggressive conditions or derivatization to elute these species, and even then such approaches are not always successful.[186-187]

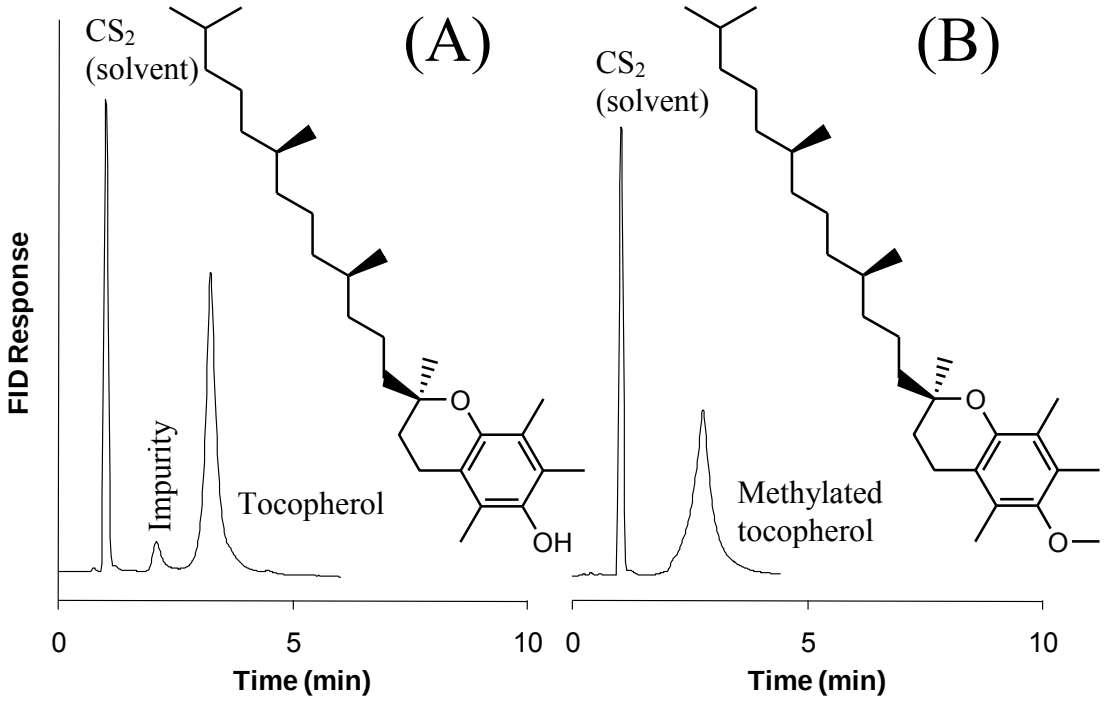


Figure 6-11: Elution of (A) tocopherol and (B) methylated tocopherol at 50 °C with 80 atm of CO_2 for 1.5 minutes followed by a 20 atm/min increase to 160 atm and 0 µL/min of water. 10 m × 250 µm I.D. stainless steel column.

Another example of an analyte with extremely limited water solubility which also demonstrates surprising retention in this system is 1-octadecanoic acid. Figure 6-12 demonstrates the elution of 1-octadecanoic acid, which is baseline resolved from the unretained CS_2 solvent. In this case, despite the eighteen carbon aliphatic chain, only the lone carboxylic acid group is required for slight retention in this system.

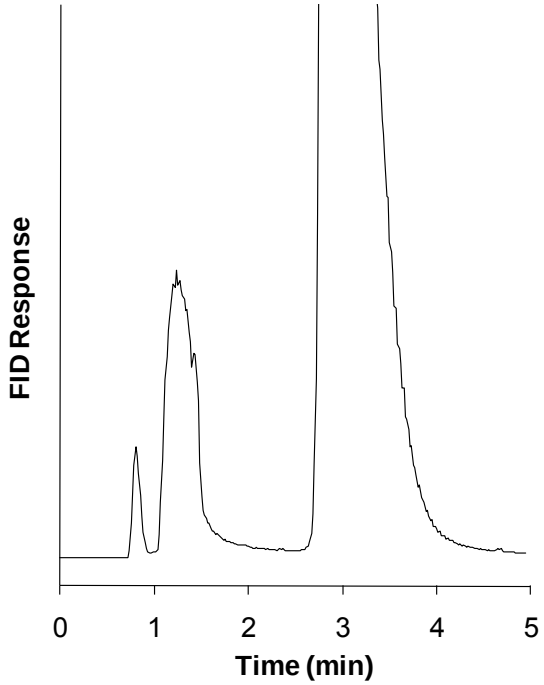


Figure 6-12: Elution of 1-octadecanoic acid at 100 °C with 130 atm of CO_2 (~220 µL/min) and 1 µL/min of water. Elution order: CS_2 (solvent), octadecanoic acid, butanoic acid (solvent). 10 m × 250 µm I.D. stainless steel column.

6.5.3 Analyte functionality

The surprising retention of octadecanoic acid and the tocopherols in the system led to further studies into which analyte functional groups could provide for retention on the water stationary phase. In this regard, Table 6-2 lists the retention times and functional groups of a wide variety of analytes eluting from the system at a constant condition of 100 °C with 80 atm of CO_2 (~190 µL/min) and 1 µL/min of water. From these data, it can be gathered that certain functional groups have a relatively larger effect on analyte retention in this system. For example, with a propane backbone, the carboxylic

acid functional group provides more retention than the alcohol which, in turn, is more retained than the aldehyde. Finally, propane diol was so heavily retained that it did not elute from the column at all within the timeframe of this experiment. This retention trend is not surprising since it follows with the water solubility of these species.

Table 6-2: Comparison of the retention times of analytes with various functional groups eluted from the system at 100 °C with 80 atm of CO_2 (~190 µL/min) and 1 µL/min of water.

Analyte	Functional Group	Retention Time (min)
1-Propanoic acid	Carboxylic acid	13.61
1-Propanol	Alcohol	2.20
1-Propanal	Aldehyde	0.80
1,2-Propanediol	Di-alcohol	No elution
1-Butanoic acid	Carboxylic acid	7.51
1-Butanol	Alcohol	1.45
1-Butanethiol	Thiol	No retention
1-Butylamine	Amine	No elution
1-Chlorobutane	Halide	No retention
Butyl ethanoate	Ester	No retention
Butyl ether	Ether	No retention
Methyl isobutyl ketone	Ketone	No retention
2-Methyl-2-butanol	Tertiary alcohol	0.81
Tributyl phosphate	Phosphoester	0.73, slightly tailed peak
Benzene	Cyclic aromatic	No retention
Pyridine	Heterocyclic aromatic	3.56, badly tailed peak

Further, when comparing functional groups on a butane backbone, a similar trend was observed. For example, the carboxylic acid group provided for greater retention over the primary alcohol, which was more highly retained than a tertiary alcohol. However, most of the other functionalized butane compounds were not retained at all in this system. In this regard, the thiol, ester, ether, ketone, and halide functional groups did not provide enough interaction with the stationary phase for retention in this system. Conversely, surprisingly high retention of butyl amine was observed in the system which did not fit the analyte trend of water solubility. In this case however, the high retention is likely explained by a reaction of the primary amine with the CO_2 mobile phase forming a carbamate salt. This type of reaction is commonly encountered while analysing amines in SFC[138-139], and could readily explain the unexpectedly high retention of butyl amine.

Next, the retention of a phosphorous-containing compound was evaluated by injecting a tributyl phosphate solution onto the column. This analyte demonstrated good retention in the system but, had a slightly tailed peak profile, perhaps hinting toward a unique solute to stationary phase interaction. In addition, the retention of pyridine was significant in the system with a badly tailed peak profile. This is surprising, since benzene is not retained at all. Clearly, the additional retention of pyridine is due to the presence of the nitrogen atom of the heterocycle. Further, the lack of retention of the butyl ether does not support the surprisingly high retention of methylated tocopherol in the system. This observation may indicate that a more complex retention mechanism than simple partitioning is responsible for retention of some analytes in this system. It should also be noted that the majority of the peak shapes observed in this system are quite symmetrical and fit very well to a conventional Gaussian distribution. As such, normally no peak

tailing is observed for analytes regardless of whether or not they contain polar functional groups with the exception of tributyl phosphate and pyridine which both resulted in a tailed peak profile. The poor peak shape of these analytes further support that another retention mechanism may be at play in addition to simple partitioning in and out of the water stationary phase. However, further work is required to verify this.

6.6 Sample Capacity

In order to test the system's sample capacity and its impact on peak shape, injections of progressively more concentrated 1-propanol solutions were made and the results are shown in Figure 6-13. The results indicate that no significant column overloading is observed for analyte peaks of up to near 50 μg of injected mass. However, peak fronting is first observed at about 100 μg of injected mass and when a large, pure injection (~400 μg) of 1-propanol is finally introduced to the system, significant peak fronting is induced. In contrast, when this experiment is repeated with 1-pentanol (a less water-soluble analyte), peak fronting is first evident at 50 μg. Finally, when injecting an analyte with even further reduced water solubility, tocopherol, peak fronting can be detected at 5 μg of injected mass. Therefore, in its current format, the system appears to have a very reasonable sample capacity for water-soluble analytes and this threshold appears to be consistently related to the analyte solubility in the stationary phase. Further, the system also appears to provide very good analyte mass balance, since no evidence of solute loss on the column was detected in routine trials.

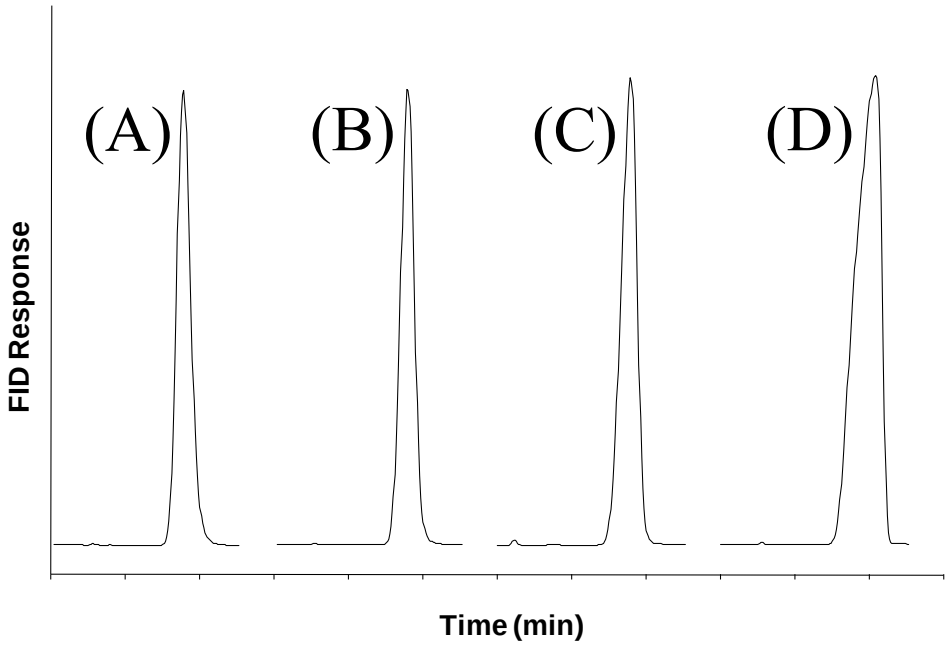


Figure 6-13: Various masses of 1-propanol injected at 100 °C with 80 atm of CO_2 (~200 µL/min) and 1 µL/min of water. The masses are (A) 5.6 µg, (B) 51.2 µg, (C) 100.2 µg, and (D) 401.7 µg (pure injection). Asymmetry values at 10% of the peak height are (A) 1.18, (B) 1.00, (C) 0.69, and (D) 0.31. Peak heights are normalized for clarity. 10 m × 250 µm I.D. stainless steel column.

6.7 Gradient Programming

To investigate the potential of gradient programming in this system, both temperature and CO_2 pressure programming were explored. Figure 6-14A and Figure 6-14B respectively demonstrate a temperature and a pressure programmed separation of a mixture of carboxylic acids. As observed in Figure 6-14A, after 5 minutes at 100 °C, a temperature program of 5 °C/min was applied to a final temperature of 150 °C (solid trace). When this programmed separation is compared to an isothermal chromatogram at

100 °C (dashed trace), a decrease in total elution time of approximately 50% is observed. Similarly, pressure programming also results in a decrease in separation time. As

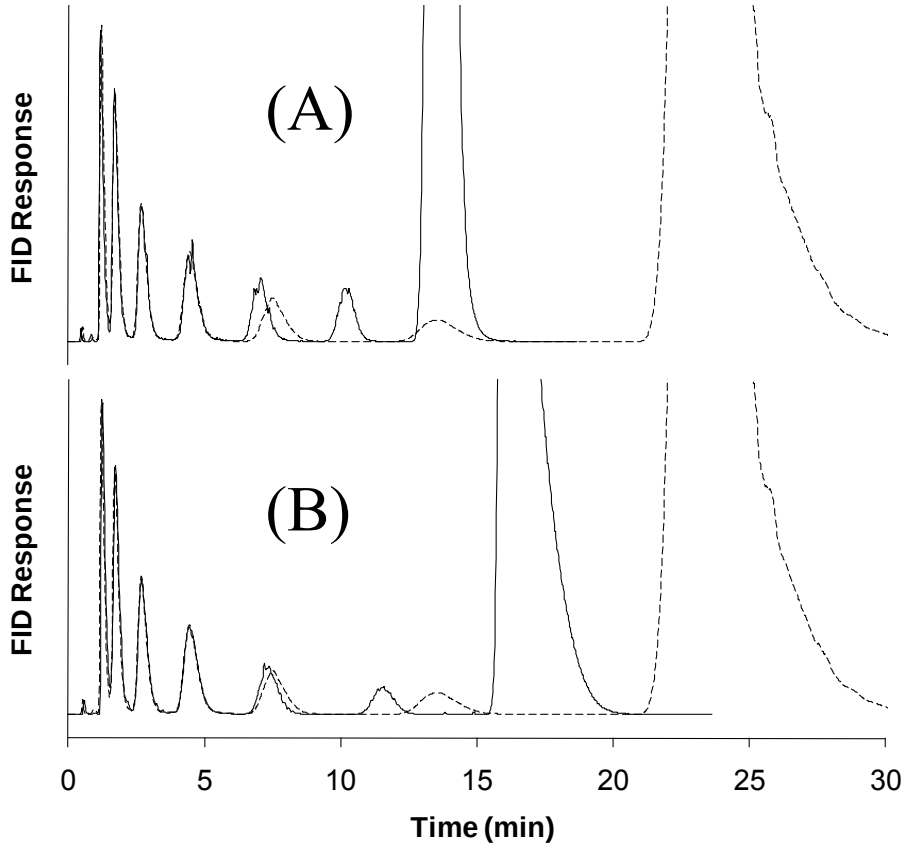


Figure 6-14: Gradient programmed separations of carboxylic acids with a water flow rate of 1 µL/min on a 10 m × 250 µm I.D. stainless steel column. (A) Solid trace: temperature program, 100 °C for 5 min, 5 °C/min to 150°C. Dashed trace: isothermal at 100 °C. Both at 80 atm CO_2 (200 µL/min). (B) Solid trace: pressure program, 80 atm CO_2 for 5 min (200 µL/min), 5 atm/min to 130 atm (350 µL/min). Dashed trace: isobaric 80 atm CO_2 (200 µL/min). Both at 100 °C. Elution order: octanoic acid, heptanoic acid, hexanoic acid, pentanoic acid, butanoic acid, propanoic acid, ethanoic acid (solvent).

observed in Figure 6-14B, a program of 5 atm/min to 130 atm after a 5 min initial period at 80 atm (solid trace) results in a 30% reduction in total elution time when compared to an isobaric separation at 80 atm of CO_2 (dashed trace). It should be noted that for the purposes of this short experiment the water flow rate was held constant at 1 μL/min. However, over longer periods of time at 150 °C, larger water flow rates would be required to maintain the phase. Overall, both temperature and pressure programming appear to operate well and present useful options here for gradient techniques to decrease analysis times.

It is noted that the acetic acid sample solvent elutes last in Figure 6-14, after the analytes elute. Still, trials with other sample solvents that elute first, such as octanol, demonstrate that analyte retention is not affected by this change. Therefore, the sample solvent does not appear to modify the retention properties of the stationary phase in advance of the analyte.

6.8 Applications

6.8.1 Alcoholic beverages

Since low molecular weight alcohols were used in exploring this system, it was natural to use this method to analyse for them in some commercially available samples. The simplest of these applications was quantifying the amount of ethanol in alcoholic beverages. For example, using a 1-propanol internal standard the alcohol content of 80-proof bourbon whiskey was readily determined to be 39.3% (versus 40% on the label) while that of a wine sample was 12.7% (versus 12.5% expected). Thus, these determined

values agree well with the anticipated quantities, and therefore the system can easily be applied to such analyses.

6.8.2 Ethanol blended gasoline

A more interesting challenge, however, is the analysis of ethanol in biofuels. A sample of such fuel was purchased locally and had an advertised content of "up to 10% ethanol by volume" (a so-called E10 biofuel). In this case, we analysed this sample in our system using external standards and determined the ethanol content to be 9.03%. The results of analysing this fuel can be found in Figure 6-15A. As seen, the system conditions are able to selectively retain and analyse ethanol from the bulk, non-polar gasoline components. This is achieved by increasing the CO_2 pressure in order to fully dissolve the gasoline components. Furthermore, since the ethanol retains some water solubility, it is retained and elutes as a lone peak after all of the unretained gasoline components. Also, as might be anticipated, when this analysis is compared to that of a gasoline containing no ethanol, under the same conditions, the ethanol peak disappears.

Figure 6-15B shows a conventional, temperature-programmed GC analysis of the same E10 biofuel gasoline sample for comparison. Also in this figure, the dashed trace represents an ethanol standard, eluted under the same conditions for peak identification purposes. As observed, conventional GC analysis of the ethanol content is more challenging, lengthy, and prone to interference by comparison. Further, benefits of the CO_2 / water system in this regard are that it performs the analysis under a constant set of conditions, whereas conventional GC requires a temperature program to elute all components with long intermittent heating and cooling cycles between trials. Also, due to

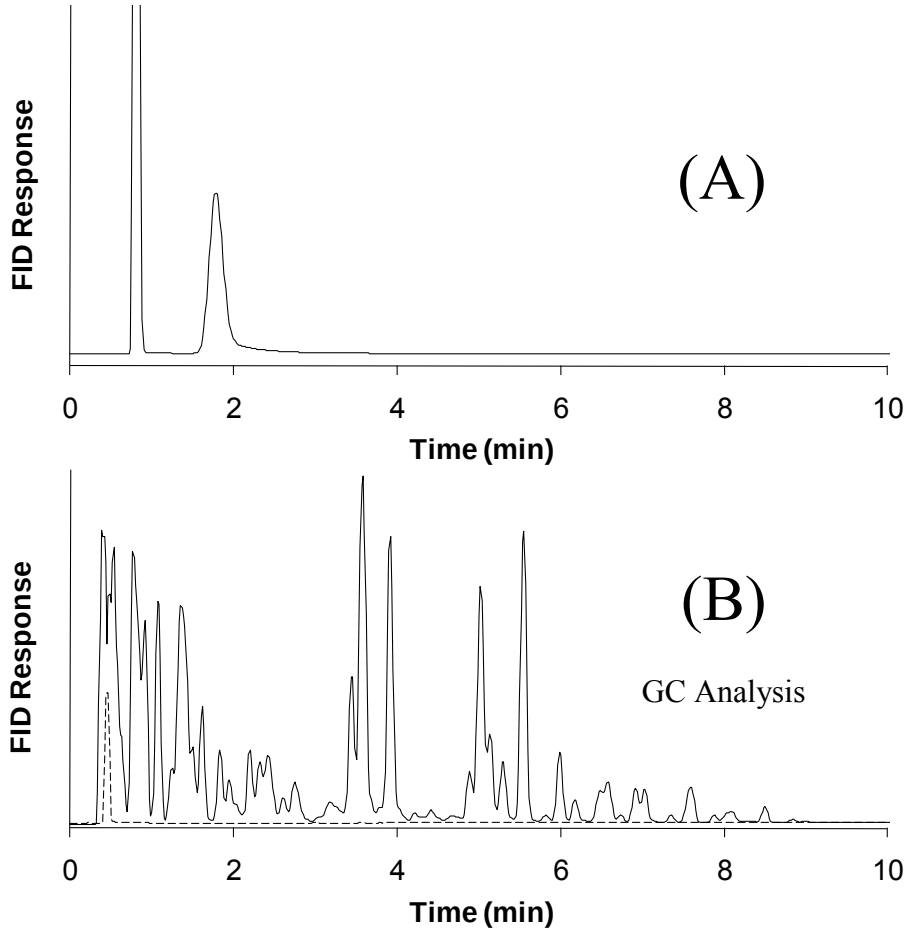


Figure 6-15: Analysis of E10 biofuel gasoline. (A) Neat E10 biofuel gasoline injected at 160 °C, 80 atm CO_2 (~290 µL/min) with 1 µL/min of water on a 10 m × 250 µm I.D. stainless steel column. Elution order: gasoline components followed by ethanol. (B) Conventional GC analysis of the same sample, diluted 10% v/v in CS_2. Temperature programmed separation starting at 45 °C and increasing upon injection at 10 °C/min to 220 °C. The dashed trace is an ethanol standard eluted under the same conditions.

the inherent properties of the CO_2 / water system and its bias against compounds insoluble in water, no sample preparation step was required for the E10 biofuel analysis. Rather, the gasoline was injected directly from the pump. Conversely, a dilution step was required for the conventional GC analysis to prevent overloading of the capillary column. Thus, such biofuels can readily and effectively be analysed by this CO_2 / water system.

6.8.3 Caffeinated beverages

The system was also applied to the analysis of caffeine levels in beverages. In this case, conditions were selected that favoured the rapid elution of caffeine from the other water soluble matrix components. A cup of coffee from a popular Canadian franchise was analysed using external standards and was found to contain 0.392 mg/mL of caffeine (versus the published value of 0.333 mg/mL). Secondly, a highly caffeinated, commercial energy beverage was analysed in a similar fashion and was found to contain 3.81 mg/mL of caffeine (versus the label value of 3.85 mg/mL). The energy beverage analysis is depicted in Figure 6-16 and further demonstrates the other extreme of the system and its inherent ability to bias against compounds insoluble in CO_2. As a result, the figure shows only a few peaks adjacent to the large caffeine response, making quantification quite straightforward and selective. This process is again further simplified by the fact that each of these beverages was sampled directly from the container and injected neat, without any sample preparation steps.

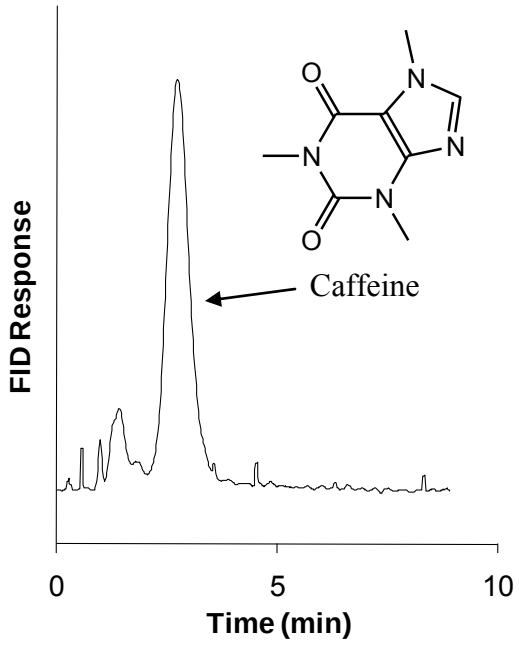


Figure 6-16: Analysis of caffeine in an energy beverage. Conditions are a neat injection at 20 °C, 200 atm CO_2 (~300 µL/min) with 0 µL/min of water. 10 m × 250 µm I.D. stainless steel column.

6.8.4 Non-ionic surfactants

Due to the successful CO_2 modified SWC separation of non-ionic surfactants in Chapter Three, it was worth investigating whether similar compounds could be analysed in the water stationary phase system. In this regard, Figure 6-17 demonstrates a Brij®-30 (polyoxyethylene (n~4) lauryl ether) separation at 100 °C with 80 atm of CO_2. As shown in this figure, although the separation was quite noisy, the constituent components were successfully separated. Therefore, this mode of chromatography may provide another possible separation system for the analysis of this class of compounds that can be difficult

to analyse in both GC (due to volatility) and HPLC (due to the lack of a UV-Vis chromophore).

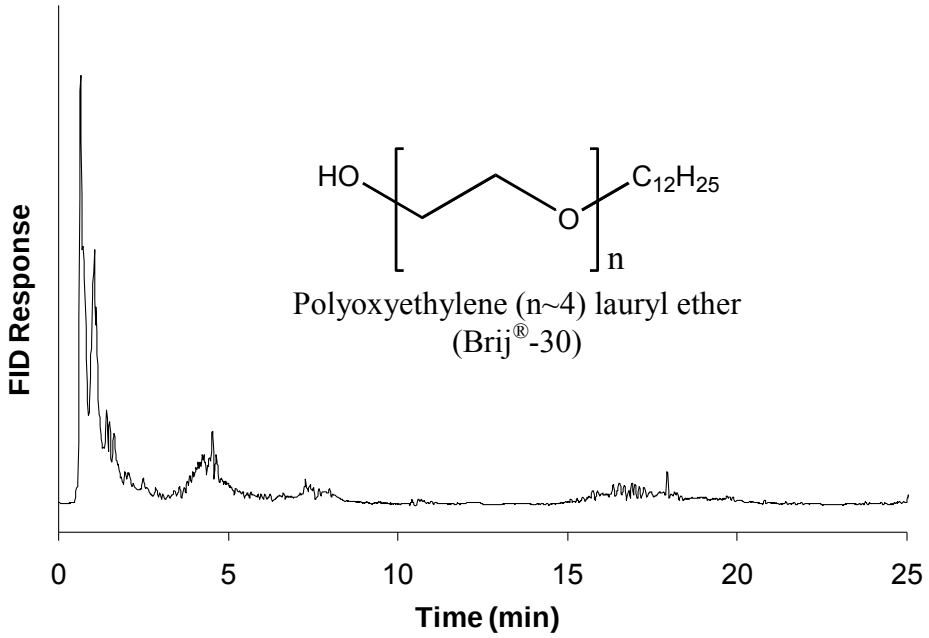


Figure 6-17: Separation of a Brij®-30 (Polyoxyethylene (n~4) lauryl ether) solution at 100 °C with 80 atm CO_2 (~185 µL/min) and 1 µL/min of water. 10 m × 250 µm I.D. stainless steel column.

6.9 Conclusions

We have presented a novel chromatographic separation method which employs a water stationary phase coating the inside of a long, bare stainless steel capillary and a CO_2 mobile phase. The separation method offers very good sample capacity, peak symmetry, and retention time reproducibility as well as many parameters for optimizing the separation such as column temperature, CO_2 pressure and CO_2 flow rate. This system

also employs only environmentally compatible, safe and inexpensive components in both the mobile and stationary phase. Lastly, due to the nature of CO_2 and water, this system is compatible with the FID which allows for inexpensive and universal detection. Overall, the results suggest that this novel separation method could be a useful alternative in a variety of separation applications.

Chapter Seven: **CHROMATOGRAPHY USING A MODIFIED WATER STATIONARY PHASE AND A CARBON DIOXIDE MOBILE PHASE**

7.1 Introduction

The system employing a water stationary phase and a CO_2 mobile phase offers a unique opportunity to alter separations since compounds can easily be dissolved in the water phase. The addition of such compounds has the potential to alter properties of the water phase such as pH, ionic strength and retention character. Therefore, changing these properties potentially could have an effect on the separation characteristics.

One such modification easily achieved is altering the pH of the stationary phase. Such pH modifications have long been employed in HPLC separations through the addition of buffers to aqueous mobile phase components. Altering the pH of the mobile phase in HPLC is often used to optimize efficiency and peak shape[188] as well as alter the retention time of acidic and basic compounds.[189] In particular, this technique is frequently employed to alter the selectivity toward weakly acidic or basic compounds without a hardware change to the system. Also, SFC often uses very acidic additives to affect the degree of solute ionization.[190] Akin to this, altering the pH of the water stationary phase has the potential to change the partitioning of a weak acid or base between the CO_2 mobile phase and water stationary phase and therefore alter retention in the system.

In addition to pH, the ionic strength of the stationary phase can also be easily altered by adding ionic compounds. In this regard, the effect of salt-loaded water stationary phases in packed column GC has been demonstrated in the literature.[166] In this study, it was shown that an increase in the amount of sodium chloride dissolved in the

water stationary phase resulted in a corresponding increase in retention for a variety of analytes. In addition to GC, ionic strength has also been extensively employed in HPLC to affect retention and column overloading behaviour.[191-192] In this regard, altering the ionic strength of the water stationary phase also has the potential to have an effect on separations employing a CO_2 mobile phase.

The water phase also could be loaded with compounds that can complex with specific analytes in order to alter selectivity. For example, mercury and silver ions have been added to water stationary phases in packed column GC.[193] In this regard, the presence of mercury results in very high retention of olefins when a non-volatile mercury complex is formed on the column. Also, the addition of silver to the GC stationary phase results in complexion with aromatics, which, in turn, results in increased retention of these compounds. Conversely, in HPLC, silica columns impregnated with silver nitrate have found success in their ability to increase selectivity when separating lipids.[194] Specifically, the addition of silver ions to the HPLC column aids in the analysis of the positional isomers of unsaturated free fatty acids.[195] In this regard, the silver ion complexes more strongly to the *cis*-isomer thereby increasing its retention and aiding in the quantitation of fatty acids in biologically relevant samples. Therefore, it is anticipated that a similar modification to the water stationary phase could alter retention of unsaturated fatty acids as well.

Another interesting potential modifier to the stationary phase is the addition of long-chain alkyl salts such as dodecylbenzene sulfonic acid (DBS). Adding long hydrophobic chains to the stationary phase could potentially change the overall character of the water phase such that non-polar compounds may be more retained. This could

potentially impart a slight reversed phase like character to the stationary phase and alter analyte retention accordingly. Since the water stationary phase is truly stationary, no interference with the FID should be expected to arise from the addition of carbon-containing compounds to the water phase.[110]

Chapter Seven explores loading the water stationary phase with various water-soluble components in efforts to change the retention of select analytes. As such, pH and ionic strength will first be altered, followed with loading the stationary phase with silver ions and concluding with the addition of dodecylbenzyl sulfonic acid to the water phase. The potential to alter the separation character of a range of model analytes will be presented and discussed. The instrumental parameters described in section 2.5 are used in these experiments.

7.2 pH Modifications

7.2.1 Model experiment

In order to demonstrate the efficacy of altering the pH of the water stationary phase, a model experiment representing the separation system was first performed. In this regard, three test tubes were prepared, each containing 8 mL of hexanes and 8 mL of an aqueous HCl solution, as shown in Figure 7-1. In this regard, the hexanes (non-polar) layer represents the CO_2 mobile phase and the HCl (polar) layer represents the modified water stationary phase of the separation system. In order to illustrate the effect of acidifying the stationary phase, the aqueous layer of the first test tube was adjusted to a pH of 2. The second tube was adjusted to a pH of 5 and the third and final tube was adjusted to a pH of 7. Next, 66.9 mg of pentanoic acid was added into each tube. As such,

since the pK_a of pentanoic acid is 4.83,[196] each tube should contain a unique distribution of pentanoic acid between the non-polar and polar layers. For example, since the first solution has a polar layer with a pH well below the pK_a of pentanoic acid, the acid should exist predominately in its protonated (i.e. neutral) state and therefore be able to partition into the non-polar layer. The second tube, containing a pH 5 HCl layer, should provide for conditions under which the pentanoic acid could exist in either the protonated or deprotonated state since the pH of solution is near to the pK_a of pentanoic acid. Therefore, pentanoic acid should exist in both the polar and the non-polar layers. Finally, the pentanoic acid in the third test tube should exist predominately in the polar layer since the pH of solution is above the pK_a of pentanoic acid, thereby forcing most of the acid into the deprotonated (i.e polar) state and preventing its partition into the non-polar layer.

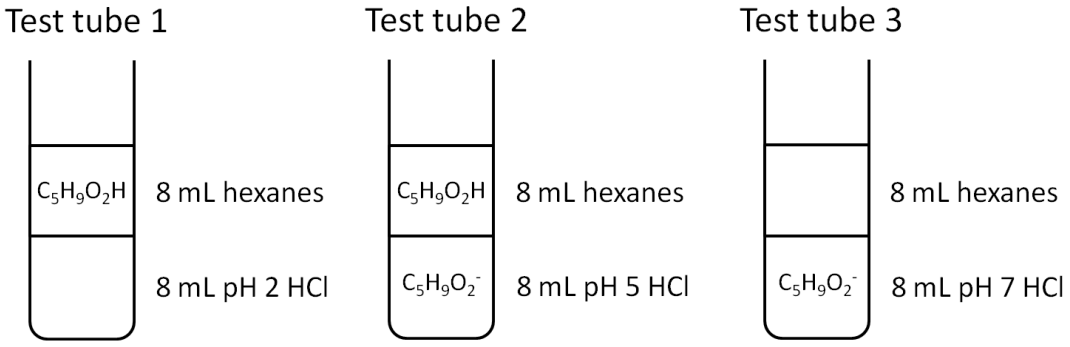


Figure 7-1: Schematic of the 1-pentanoic acid partitioning experiment. 66.9 mg of 1-pentanoic acid was added to each tube. The expected presence and ionization state of pentanoic acid in each layer indicated by its chemical formula.

In order to evaluate in what phase the pentanoic acid resides, the polar and non-polar layers of each test tube were analysed with a 10 m column in the separation system employing a water stationary phase and a CO_2 mobile phase. The results of these analyses are shown in Figure 7-2. Figure 7-2A and Figure 7-2B represent the non-polar phase and polar layers of the first (pH 2) test tube, respectively. As seen here, most of the pentanoic acid resides in the non-polar layer, confirming that the low pH condition is

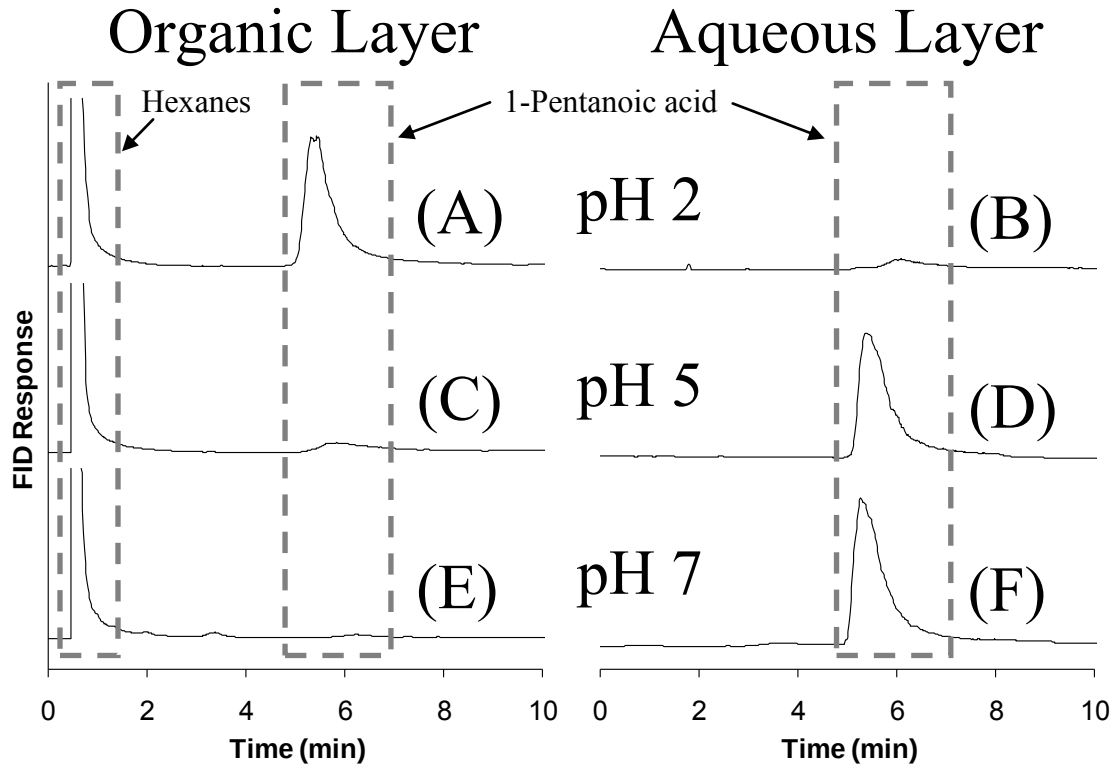


Figure 7-2: Analysis of the solutions prepared as per Figure 7-1 at 100 °C with 80 atm of CO_2 (~200 µL/min) and 1 µL/min of water. 10 m × 250 µm I.D. stainless steel column. (A) is the pH 2 organic layer, (B) is the pH 2 aqueous layer, (C) is the pH 5 organic layer, (D) is the pH 5 aqueous layer, (E) is the pH 7 organic layer, and (F) is the pH 7 aqueous layer.

protonating the acid and allowing for it to partition into the non-polar layer. Next, Figure 7-2C and Figure 7-2D represent the non-polar layer and polar layer of the second (pH 5) test tube, respectively. As seen, most of the pentanoic acid resides in the polar layer, with a small portion in the non-polar layer. This confirms that the solution pH just above the pK_a of hexanoic acid provides for a condition that allows the acid to exist in both the protonated and deprotonated state and therefore exist in each layer of the test tube. Finally, Figure 7-2E and Figure 7-2F represent the non-polar layer and polar layer of the third (pH 7) test tube, respectively. In this case, most of the pentanoic acid resides in the polar layer, confirming that the higher pH condition is deprotonating the acid and preventing it from partitioning into the non-polar layer.

Overall, these simple experiments verify two main things. First, modifying the pH of the solution has the ability to change the extent of analyte solubility in the non-polar layer. Since the non-polar layer in these experiments is an analogue of the CO_2 mobile phase, these model experiments could potentially help predict the behaviour of test analytes in the separation system employing a pH-modified water stationary phase. For example, reducing the pH of the stationary phase could protonate an analyte anion and increase the solubility of the analyte in CO_2 and therefore reduce its retention time on the column. Second, and more interestingly, these experiments controlled the form (i.e. charged or neutral) of the analyte prior to injection in the system. Couple to this the fact that peaks have been detected from analytes that should be negatively charged (i.e. carboxylic acids prepared in neutral water) and therefore very highly retained in the stationary phase (i.e. not eluted). Therefore, this suggests that the interface of CO_2 and water in the system may be fairly acidic as has been reported previously.[197-199]

7.2.2 Practical evaluation

Since the model experiment indicates that pH could affect the solubility of an analyte in the CO_2 mobile phase, investigations were next performed to evaluate what effect of altering the pH of the stationary phase has on the retention of model analytes. The first of such tests was performed using a citric acid test analyte. As noted earlier in Chapter Six, citric acid could not be eluted at all from a column containing a pure water stationary phase. Therefore, it was thought that citric acid could have higher CO_2 solubility if it was forced into its fully protonated (i.e. neutral) state. In this regard, the resultant higher solubility of protonated citric acid in CO_2 might therefore allow for the elution of this form of citric acid from the column. In order to fully protonate citric acid, a stationary phase would have to be prepared with a pH less than the citric acid pK_{a1} value of 3.13.[200] In this regard, a pH 1 HCl stationary phase was prepared and established on the column. Figure 7-3A shows the attempted elution of the citric acid solution from a 1 m column with the pH 1 HCl stationary phase. As shown in this figure, citric acid still could not be eluted from the column in a reasonable amount of time even with a highly acidic stationary phase.

Next, it was considered that, since citric acid was prepared in water, the injection of 0.5 μL of water (with the analyte) onto the column could have a significant localised dilution effect of the stationary phase. Considering that a 1 m column only contains about 3.3 μL of stationary phase, perhaps the water injection caused a localized region of higher pH at the head of the column which provided for a region that did not allow the full protonation of citric acid. In order to address this concern, a second citric acid sample was prepared in pH 1 HCl. Figure 7-3B depicts the elution attempt of this acidified citric

acid solution. Again, unfortunately, no elution of a citric acid peak was observed in a reasonable timeframe. The lack of citric acid elution from the modified water stationary phase system, even in its fully protonated form, may be because it remains a very polar molecule, likely with limited CO_2 solubility in the neutral form.

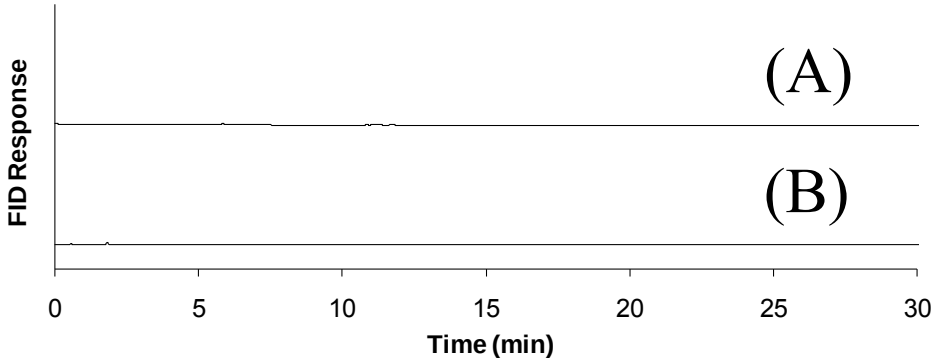


Figure 7-3: No elution of citric acid from a pH 1 HCl stationary phase at 20 °C with 100 atm of CO_2 (~130 µL/min). The citric acid was prepared in (A) water and (B) pH 1 HCl. 1 m × 250 µm I.D. stainless steel column.

The effect of acidifying the stationary phase was evaluated next using a test analyte with considerably less polar functionality. DBS was selected due to its long alkyl moiety (Figure 7-4) which should provide for good CO_2 solubility as supported by previous, successful separations of DBS in SFC.[201-202] However, DBS is considerably more acidic than citric acid and therefore, to ensure protonation, a more highly acidic stationary phase was prepared. As such, a pH 0 HCl stationary phase was prepared and established in the column. In addition, to help prevent stationary phase dilution, the DBS sample was prepared in pH 0 HCl as well.

Figure 7-4: Structure, pK_a value, and molecular weight of dodecylbenzene sulfonic acid.

Since increasing both temperature and pressure have been shown to significantly increase the solubility of sulfonates in supercritical CO_2,[203] a range of different separation conditions were attempted for the elution of DBS, as outlined in Table 7-1. Unfortunately, none of these conditions, which spanned CO_2 pressures from 100 to 260 atm and column temperatures from 20 °C to 150 °C, resulted in elution of DBS from the column. For example, Figure 7-5A shows an unsuccessful elution attempt of DBS at 20 °C with 100 atm of CO_2. However, it should be noted that, although the high temperatures and pressures used would readily evaporate the stationary phase over longer amounts of time, the timeframe of these separations should be short enough that this should not be an issue. In addition, since no DBS was eluted, the stationary phase must have remained intact throughout these trials.

Table 7-1: Summary of conditions attempted for the elution DBS from a pH 0 HCl stationary phase.

Column Temperature (°C)	CO_2 Pressure (atm)	CO_2 Flow Rate (µL/min)	DBS Elution?
20	100	70	No
20	200	40	No
20	200	40	No
20	200	140	No
50	260	160	No
150	260	110	No

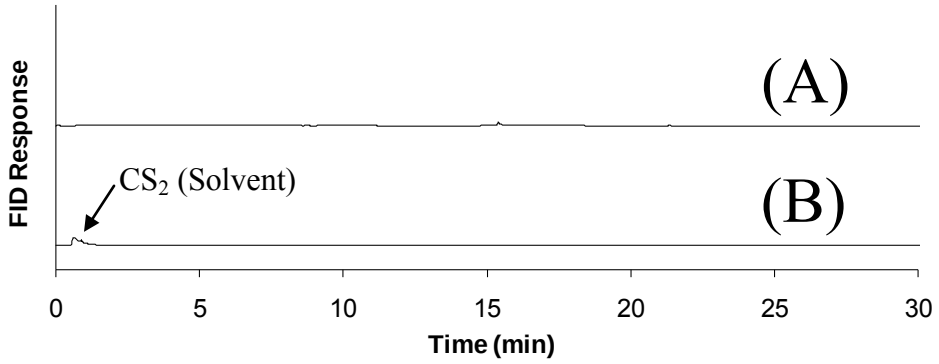


Figure 7-5: No elution of DBS from a pH 0 HCl stationary phase at 20 °C with 100 atm of CO_2 (~70 µL/min). The DBS was prepared in (A) water and (B) CS_2. 1 m × 250 µm I.D. stainless steel column.

Additionally, since DBS is soluble in non-polar solvents, a second sample was prepared in CS_2. In this regard, no chance of stationary phase dilution is possible due to the extremely limited solubility of water and CS_2. However, even when the possibility of stationary phase dilution was completely eliminated, no DBS peak was observed eluting

from the system, as shown in Figure 7-5B. Therefore, it appears as though acidifying the stationary phase is unable to provide for conditions under which DBS is completely soluble in CO_2, and therefore, no elution of the DBS peak was observed.

The next analyte employed to evaluate the acidified stationary phase was *L-α*-phosphatidylcholine (Figure 7-6). Such phospholipids are often challenging to analyse due to their occurrence in very complex biological matricies and their lack of a strong chromophore for UV/Vis detection. In that regard, ELSD or mass spectrometric detection is often required for the analysis of these biologically important molecules. Nonetheless, phospholipids have been successfully analysed by CO_2 SFC employing ELSD and mass spectrometric detection.[204-205] Borrowing from one such published SFC method, the phospholipid solution was injected onto the pH 0 stationary phase at 40 °C with 120 atm of CO_2.[205] Unfortunately, as shown in Figure 7-7, no elution was observed. This may be because it is zwitterionic and maintains a charge at the conditions used. Regardless, the acidified stationary phase was unable to provide for conditions under which *L-α*-phosphatidylcholine could be eluted from this system.

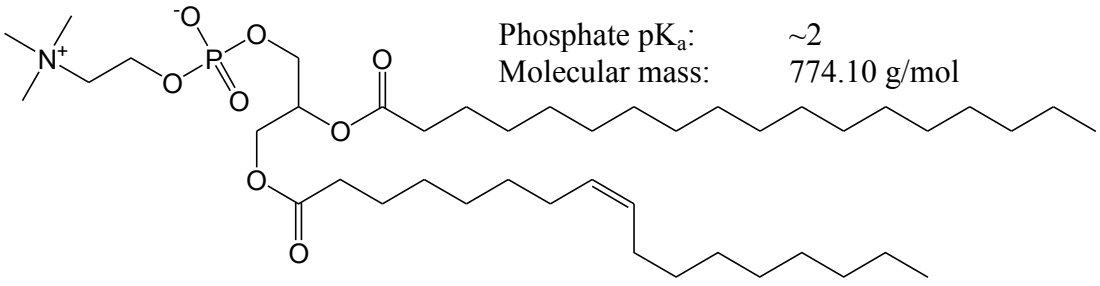


Figure 7-6: Structure, pK_a value, and molecular weight of *L-α*-phosphatidylcholine.

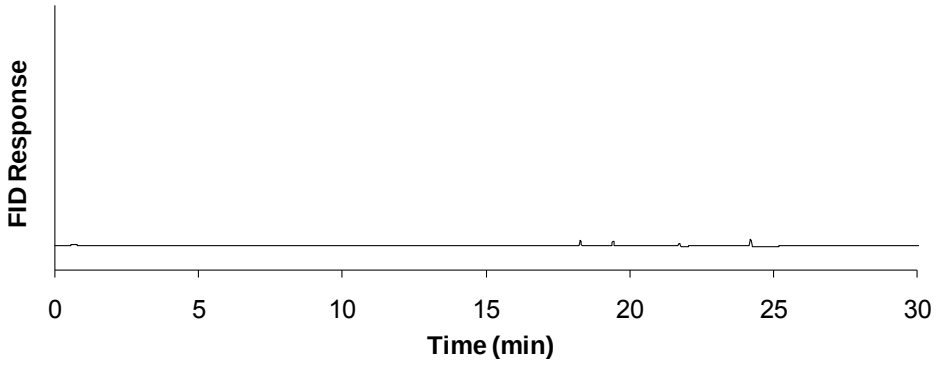


Figure 7-7: No elution of *L*-*α*-phosphatidylcholine from a pH 0 HCl stationary phase at 40 °C with 120 atm of CO_2 (~90 µL/min). 1 m × 250 µm I.D. stainless steel column.

Finally, the effect of raising the pH of the stationary phase was evaluated with a hexanoic acid test analyte. Hexanoic acid was selected since it readily elutes from the system with a pure water stationary phase. However, it was postulated that, if the pH of the stationary phase was sufficiently raised, hexanoic acid would deprotonate, thereby significantly reducing its solubility in CO_2 and increasing its retention time. Since the pK_a of hexanoic acid is 4.85,[200] a pH 12 NaOH stationary phase was employed to help ensure conditions for the deprotonation of this acid. Figure 7-8A shows the elution profile of the hexanoic acid solution from a pure water stationary phase. Conversely, Figure 7-8B shows the elution profile of hexanoic acid from the pH 12 stationary phase. As shown, no dramatic difference in the elution profile is again noted. However, the appearance of a more retained, small shoulder on the peak eluting from the pH 12 stationary phase was seen. Unfortunately though, this was the only observation noted. The origin of this feature is not yet known and more examination is required to verify its

identity. Regardless, either way, altering the pH of the stationary phase did not appear to have a measurable change on analyte retention in this separation system.

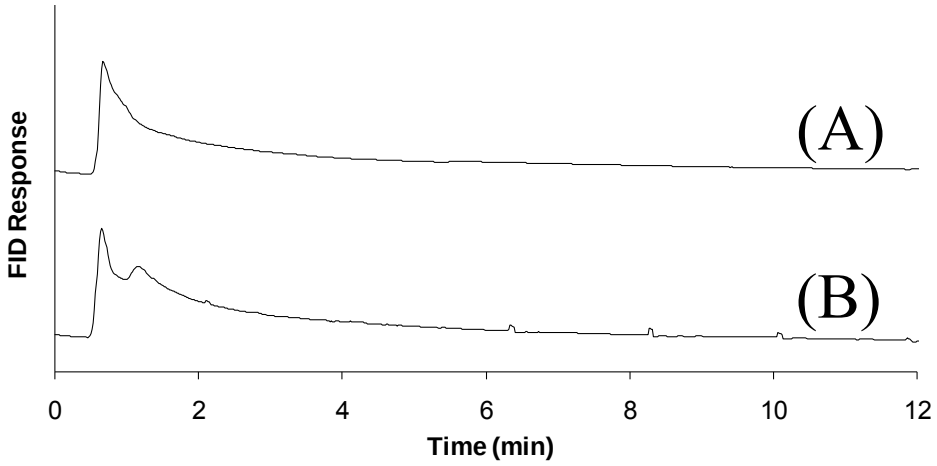


Figure 7-8: Elution of 1-hexanoic acid from (A) a water stationary phase and (B) a pH 12 NaOH stationary phase, each at 20 °C with 100 atm of CO_2 (~80 µL/min). 1 m × 250 µm I.D. stainless steel column.

7.3 Ionic Strength Modifications

Since altering the pH of the stationary phase showed no significant change in the elution pattern of test analytes, altering the ionic strength of the stationary phase was next evaluated. The n-alcohol mixture from Chapter Six was used first to investigate the effect ionic strength may have on separations in this mode of chromatography. As such, stationary phases containing 0.01 M, 0.1 M, and 1.0 M of KNO_3 were prepared. Separations of the n-alcohol mixture were performed on each stationary phase at 20 °C with 80 atm of CO_2 as summarised in Table 7-2. As shown in this table, no significant change in the retention time of the alcohol mixture was noted with either 0.01 M or 0.1 M

of KNO_3 in the stationary phase. However, the stationary phase containing 1.0 M of KNO_3 proved troublesome since it resulted in the rapid plugging of the system restrictor. As such, no separation could be performed under this condition. Further, Figure 7-9

Table 7-2: Summary of the methanol retention times for the elution of the n-alcohol test mixture from stationary phases with varying ionic strength.

KNO_3 Stationary Phase Concentration (M)	Methanol Retention Time (min)
0	2.25
0.01	1.95
0.1	2.28
1.0	N/A – restrictor plugged

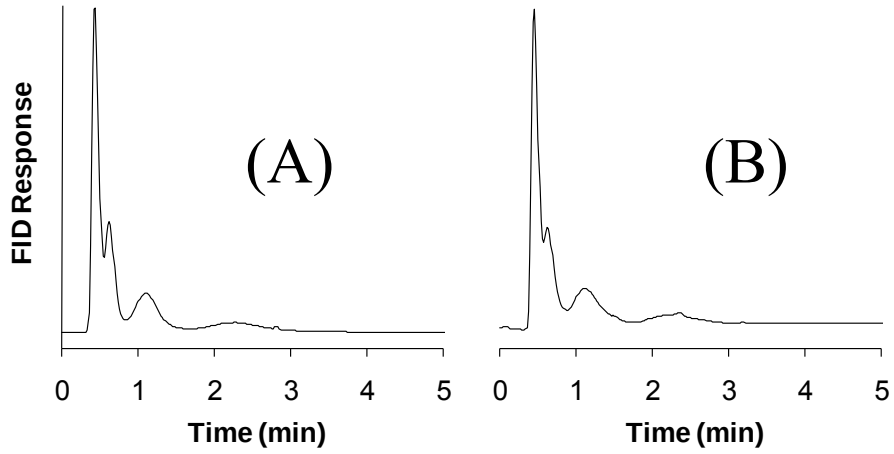


Figure 7-9: C_1-C_5 n-alcohol separation on (A) a water stationary phase and (B) a 0.1 M KNO_3 stationary phase, each at 20 °C with 80 atm of CO_2 (~280 µL/min). Elution order: pentanol and butanol co-eluting, propanol, ethanol, methanol. 1 m × 250 µm I.D. stainless steel column.

demonstrates a comparison of the elution pattern of the n-alcohol mixture from a water stationary phase (Figure 7-9A) and the 0.1 M KNO_3 (Figure 7-9B). As seen in Figure 7-9, no significant change in retention time or elution profile of the alcohol mixture was observed when comparing separations performed on the water stationary phase and the 0.1 M KNO_3 stationary phase. As such, it appears as though changing the ionic strength of the stationary phase does not have an observable effect on the separation of the n-alcohol mixture.

A second experiment was performed to evaluate the effect of the ionic strength of the stationary phase by using a DBS test analyte. DBS was selected since changes in ionic strength have been shown to have an effect on the retention time of weak acids in HPLC,[206] as well as alter the ionization of analytes in SFC.[190] A comparison was performed between a pure water stationary phase (Figure 7-10A) and a 0.1 M KNO_3

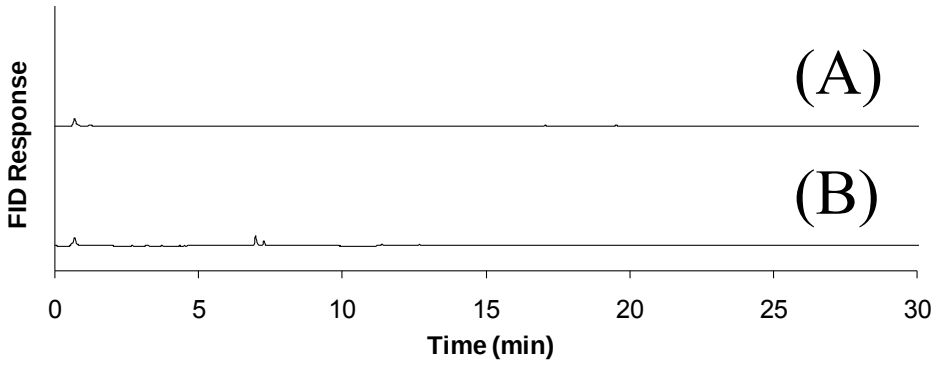


Figure 7-10: Elution of DBS acid from (A) a water stationary phase and (B) a 0.1 M KNO_3 stationary phase, each at 20 °C with 80 atm of CO_2 (~155 µL/min). 1 m × 250 µm I.D. stainless steel column.

stationary phase (Figure 7-10B). As shown in this figure, no elution of DBS was observed from either column. In this regard, altering the ionic strength of the stationary phase does not appear to allow for DBS elution from the column.

7.4 Silver Ion-Loaded Stationary Phase

Free fatty acids present a separation challenge in many modes of chromatography. For example, prior to analysis in GC, derivitazation to a fatty acid methyl ester is often required to increase analyte volatility. In addition, the lack of a strong chromophore prevents the use of UV/Vis in HPLC for the analysis of free fatty acids. Finally, separating the *cis*- and *trans*- geometric isomers of free fatty acids can be challenging since resolving these similar compounds is often difficult. However, in order to aid in the resolution of geometric isomers of free fatty acids, HPLC stationary phases impregnated with silver salts are often employed. In this regard, the *cis*-isomer becomes more highly retained than the *trans*-isomer or the saturated form, thereby aiding in separation.[195] Therefore, the addition of silver ions to the water stationary phase could potentially provide an alternate method for the separation of such geometric isomers. As such, the ability of silver ions in the water stationary phase to affect separations of geometric isomers of free fatty acids was next evaluated. In this regard, a 0.1 M $AgNO_3$ stationary phase was prepared and comparative separations of stearic acid (saturated octadecanoic acid) and oleic acid (*cis*-monounsaturated octadecenoic acid) were performed on a 2 m column containing either a water stationary phase or the 0.1 M Ag^+ stationary phase. Figure 7-11 demonstrates these comparative separations at 20 °C with 80 atm of CO_2.

Figure 7-11A is the elution pattern of the stearic acid solution injected onto a water stationary phase. As expected, since silver ions do not interact with the fully saturated stearic acid, no change in the elution pattern was observed when the same

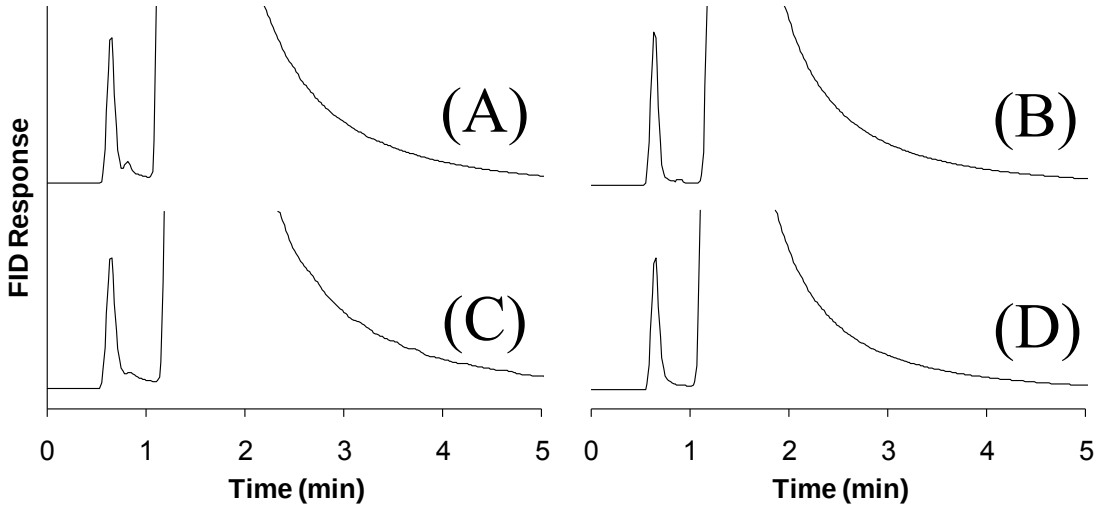


Figure 7-11: Elution of free fatty acids at 20 °C with 80 atm of CO_2 (~160 µL/min) from a 1 m × 250 µm I.D. stainless steel column. (A) stearic acid and a water stationary phase, (B) oleic acid and a water stationary phase, (C) stearic acid and a 0.1 M $AgNO_3$ stationary phase, and (D) oleic acid and a 0.1 M $AgNO_3$ stationary phase. Elution order: free fatty acid, acetic acid (solvent).

sample was injected onto the 0.1 M Ag^+ column (Figure 7-11C). Conversely, when comparing injections of the *cis*-monounsaturated oleic acid solution on the water stationary phase (Figure 7-11B) to the Ag^+ stationary phase (Figure 7-11D), a change in retention time should be expected since silver ions complex with the *cis*-bond which should, in turn, increase the retention time of this analyte on the column. However, as

shown in the figure, no change in retention time was observed. Therefore, it appears as though silver ions cannot alter the separation profile of free fatty acids in this mode of chromatography.

7.5 Alkyl Chains in the Stationary Phase

Since the efforts attempting to elute DBS from the system earlier in this chapter were unsuccessful under a wider range of conditions, it can be reasonably assumed that DBS cannot be eluted at all. Therefore, DBS would make an excellent candidate for a stationary phase additive. For example, the addition of DBS could potentially decrease the overall polarity of the stationary phase, thereby imparting a slight reversed phase character. In this regard, the retention time of injected analytes could potentially be altered.

The first mixture employed to evaluate the DBS stationary phase was the n-alcohol mixture previously used in Chapter Six. Stationary phases containing 0.0001 M, 0.001 M, 0.01 M, and 0.1 M of DBS were prepared and n-alcohol separations were performed on each at 20 °C with 80 atm of CO_2. The retention time of methanol on each stationary phase is outlined in Table 7-3. As represented by the methanol retention time data in this table, no significant change in elution time of methanol was observed until the 0.1 M DBS stationary phase case, a very subtle increase in methanol retention is observed. Further, Figure 7-12 shows this subtle increase in retention time with comparative n-alcohol separations on a water stationary phase (Figure 7-12A) and the 0.1 M DBS stationary phase (Figure 7-12B). Therefore, the addition of a large amount of

DBS to the stationary phase may potentially affect the elution profile of the mixture of n-alcohols in this mode of chromatography. However, the effect is small at best.

Table 7-3: Summary of the methanol retention times for the elution of the n-alcohol test mixture from stationary phases containing varying concentrations of DBS.

DBS Stationary Phase Concentration (M)	Methanol Retention Time (min)
0	3.42
0.0001	3.76
0.001	3.40
0.01	3.70
0.1	3.85

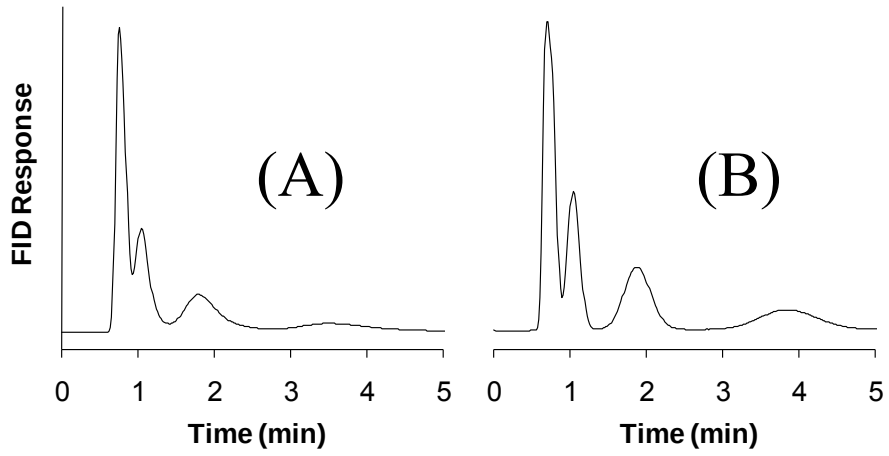


Figure 7-12: C_1-C_5 n-alcohol separation on (A) a water stationary phase and (B) a 0.1 M DBS stationary phase, each at 20 °C with 80 atm of CO_2 (~160 µL/min) and a 1 m × 250 µm I.D. stainless steel column. Elution order is the same as Figure 7-9.

Next, a mixture of light carboxylic acids was employed to further evaluate the DBS stationary phase. In a similar fashion to the n-alcohol trials, separations of the light carboxylic acid mixture were performed on the 0.0001 M, 0.001 M, 0.01 M, and 0.1 M DBS stationary phases at 20 °C with 80 atm of CO_2. The retention time of acetic acid on each stationary phase is outlined in Table 7-4. As represented by the data in this table, no significant change in elution time of the mixture was observed with stationary phases containing up to 0.01 M of DBS. Again in this case, akin to the trend observed with the

Table 7-4: Summary of the acetic acid retention times for the elution of the light carboxylic acid mixture from stationary phases containing varying concentrations of DBS.

DBS Stationary Phase Concentration (M)	Acetic Acid Retention Time (min)
0	4.50
0.0001	4.28
0.001	4.23
0.01	4.88
0.1	6.17

n-alcohols, a subtle increase in acetic acid retention is observed when the separation was performed on the 0.1 M DBS stationary phase. Figure 7-13 depicts this subtle increase in retention time with comparative short chain carboxylic acid separations on a water stationary phase (Figure 7-13A) and the 0.1 M DBS stationary phase (Figure 7-13B). Therefore, again, the addition of a large amount of DBS to the stationary phase

potentially demonstrates an effect on the elution profile of the mixture of short chain carboxylic acids in this mode of chromatography.

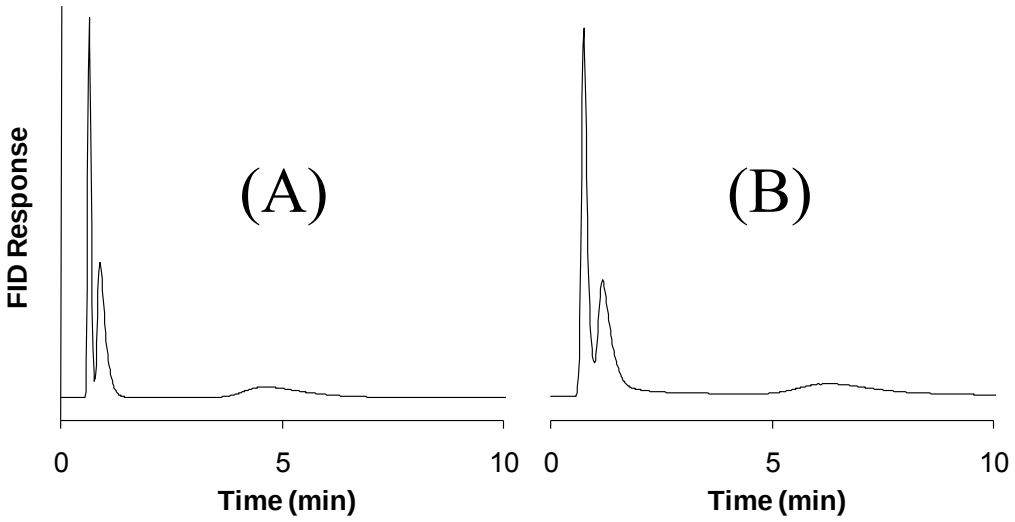


Figure 7-13: Carboxylic acid separation on (A) a water stationary phase and (B) a 0.1 M DBS stationary phase, each at 20 °C with 80 atm of CO_2 (~160 µL/min) and a 1 m × 250 µm I.D. stainless steel column. Elution order is hexanoic acid, butanoic acid, acetic acid.

Finally, a dodecanoic acid solution prepared in methanol was employed to also evaluate the DBS stationary phase. This analyte has significantly reduced water solubility relative to the other analytes employed. Again, as observed with the alcohols and short chain carboxylic acids, a slight increase in retention time is observed for the elution of the dodecanoic acid sample from the 0.1 M DBS stationary phase. Table 7-5 summarises the dodecanoic acid retention time for the elution of this sample from a water stationary phase as well as the 0.001 M and 0.1 M DBS stationary phases. Although this tabulated

data demonstrates only a very small increase in dodecanoic acid retention, the effect is more pronounced when observed visually. In this regard, Figure 7-14 shows the increase in retention time of the elution of dodecanoic acid in methanol when comparing the water stationary phase (Figure 7-14A) to the 0.1 M DBS stationary phase (Figure 7-14B). This increase in retention time on the 0.1 M DBS stationary phase also may suggest that the addition of a large amount of DBS to the stationary phase can potentially alter the elution profile of analytes in this mode of chromatography.

Table 7-5: Summary of the dodecanoic acid retention times for the elution of the dodecanoic acid test mixture from stationary phases containing varying concentrations of DBS.

DBS Stationary Phase Concentration (M)	Dodecanoic Acid Retention Time (min)
0	0.67
0.001	0.65
0.1	0.73

Although there was significant variability in the retention time of analytes on the water stationary phase containing DBS, one trend was observed for all of the test analytes. In this regard, it appeared as though all of the examples showed slightly higher analyte retention when the 0.1 M DBS stationary phase was employed. Although this shift in retention could be explained by a change in the stationary phase polarity as discussed above, other explanations can also describe the difference in retention times.

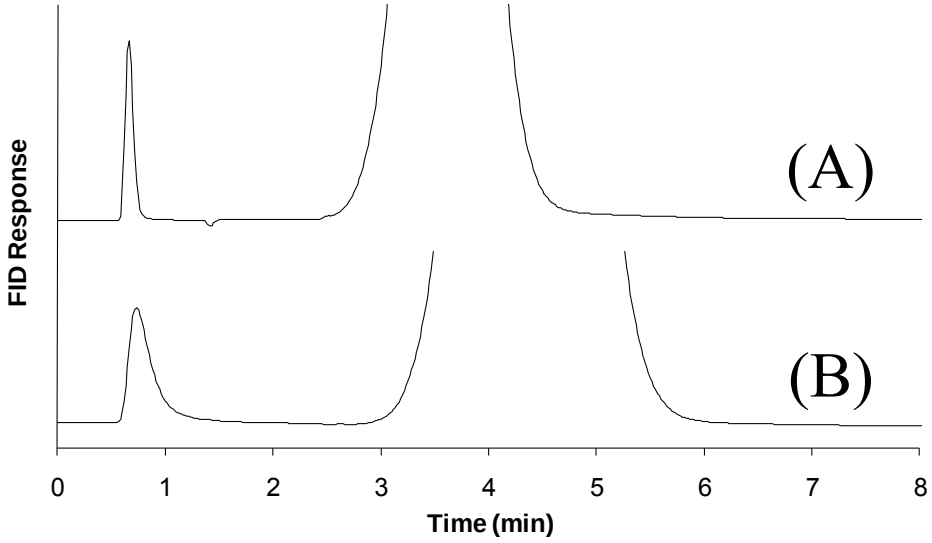


Figure 7-14: Elution of 1-dodecanoic acid from (A) a water stationary phase and (B) a 0.1 M DBS stationary phase, each at 20 °C with 80 atm of CO_2 (~160 µL/min) and a 1 m × 250 µm I.D. stainless steel column.. Elution order is dodecanoic acid, methanol (solvent).

One such explanation may be that, since DBS is a surfactant, micelle formation may be occurring around the analyte molecules. As such, micelle formation would promote better solubility of these analytes in the water stationary phase, thereby increasing their retention time. Since the critical micelle concentration of DBS is 0.01 M,[207] the 0.1 M DBS stationary phase is the only such stationary phase employed with a concentration high enough to allow formation of micelles. This potentially explains the observation that the retention time shift is only seen when the 0.1 M DBS stationary phase. Conversely, a second explanation for the shift in retention time observed with the 0.1 M DBS stationary phase is that, the presence of such a large amount of compound in the water stationary phase could be slightly occluding the system restrictor. In this regard, the CO_2 flow rate

could be potentially reduced, which, in turn, would result in an increase in analyte retention time. However, no large difference in the flow rate at the pump was noticed while operating with the 0.1 M DBS stationary phase. Therefore, more work is needed to verify the existence of this effect.

7.6 Conclusions

Although a model experiment representing the separation system employing a pH-modified water stationary phase and a CO_2 mobile phase demonstrated the ability of an acidic analyte to partition between a polar and a non-polar phase with a change in pH, no such change in pH was observed when separations were performed in a similar manner with a pH-modified stationary phase. It is not yet known whether the pH of the CO_2 / water interface impacts this. In addition, no change was observed in the elution patterns of compounds injected after changing the ionic strength of the stationary phase, or when silver ions were added to the stationary phase as complexing agents. However, perhaps a small effect on analyte retention was observed when large amounts of long alkyl chains (DBS) were added to the stationary phase. In this regard, perhaps these seminal experiments offer more information for further development into modified water stationary phases employed with this mode of chromatography.

Chapter Eight: **SUMMARY AND FUTURE WORK**

8.1 Summary

This thesis describes the successful development and exploration of novel separation systems employing carbon dioxide and water. Such systems can provide an alternative to conventional separation methods, many of which can employ toxic, expensive, and environmentally hazardous separation components. As such, water and carbon dioxide represent possible mobile phase and stationary phase alternatives which are inexpensive, safe and environmentally compatible. Further, since both are unresponsive in a FID, this inexpensive and universal detector is available for these novel separation techniques.

One such novel separation method was developed which employs non-polar CO_2 as a mobile phase additive to conventional purely aqueous SWC. The addition of CO_2 results in a significant reduction of the polarity of the mobile phase while maintaining the primary features of environmental and FID compatibility that SWC is known for. The resultant increase in elution strength was demonstrated by comparative separations employing the mixed mobile phase to a conventional, pure water SWC mobile phase. In this regard, a significant reduction in elution time was observed for compounds highly retained in conventional SWC such as acetophenones, certain alcohols, aniline derivatives, carboxylic acids, and a BTEX mixture. In addition, successful separations of compounds were demonstrated which had never before been analysed by SWC such as free fatty acids and non-ionic surfactants. As such, this method greatly extends the ability of SWC to analyse non-polar analytes.

Further, another feature resulting from the addition of CO_2 to the mobile phase is the reduction of the operating temperatures required to elute analytes separated in conventional purely aqueous SWC. As such, this feature potentially allows for the analysis of thermally labile analytes which have previously been incompatible with SWC due to thermal instability. The attempted expansion of this separation system to the analysis of pharmaceutically relevant compounds, many of which are thermally labile, was presented with the exploration of the model pharmaceutical, fluoxetine. In this regard, elution attempts were performed with both a purely aqueous mobile phase and with a mixed mobile phase from a multitude of stationary phases. Although no successful separation method was developed for fluoxetine, these experiments demonstrated this molecule's high affinity for reversed phase stationary phases in SWC and lay a good framework for future applications of this novel, CO_2 modified SWC system to pharmaceutically relevant analytes.

In addition, studies were also performed to evaluate the ability of ultrasonic radiation to further reduce the operating temperatures employed in this mode of chromatography. These studies show that ultrasound has the potential ability to promote better mixing of the CO_2 and water components of the mobile phase in the CO_2 modified subcritical water system. This is demonstrated by the noticeable improvement in the peak shape and decrease in the peak splitting observed for a test analyte. Although the peak splitting was not completely eliminated, it appears that the experiments performed in this chapter show some promise for ultrasound in this mode of chromatography, perhaps with a stronger source of ultrasound.

This thesis also presents a novel chromatographic separation method which employs a water stationary phase coating the inside of a long, bare capillary and a CO_2 mobile phase. This separation method employs only environmentally compatible, safe and inexpensive components in both the mobile and stationary phase which are both compatible with the FID. The system offers very good sample capacity, peak symmetry, and retention time reproducibility as well as many parameters for optimizing the separation such as column temperature, CO_2 pressure and CO_2 flow rate. The utility of this system was demonstrated by the analysis of ethanol in alcoholic beverages and biofuels, through the analysis of caffeine in beverages and by the separation of non-ionic surfactants.

Since this separation technique employs a water stationary phase, water soluble compounds can easily be added to change the properties of the stationary phase. As such, the ability of dissolved compounds to affect separations was also evaluated. Changes in the stationary phase pH and ionic strength as well as the addition of complex-forming ions and alkyl chains to the stationary phase were evaluated. In this regard, a noticeable change in retention behaviour was demonstrated upon addition of DBS as a stationary phase modifier. Therefore, perhaps these seminal experiments hold promise for further development into modified water stationary phases employed with this mode of chromatography.

8.2 Future Work

8.2.1 Dielectric constant of water

In SWC the primary means to lower the dielectric constant of the mobile phase is by increasing the column temperature. Although temperature is effective at altering the eluent strength, the maximum temperature available (and therefore the speed of separation) is limited by the thermal maximum of the stationary phase. In this regard, the thermal stability of the stationary phase often prevents the analysis of certain analytes which require a very low mobile phase dielectric constant for timely elution. Therefore, an alternate method of reducing the mobile phase polarity would be of great benefit to SWC. One such possible alternative to decrease the dielectric constant of water is the application of radio wave radiation.[208] As shown in Figure 8-1, the dielectric constant of water falls rapidly with an increase in radio wave frequency without an increase in temperature. For example, a radio frequency of approximately 20 GHz applied to 25 °C water results in a dielectric constant mimicking that of room temperature acetonitrile.[29] In this regard, applied radio waves could help lower the operating temperatures of SWC. Further, since a minimum temperature of 75 °C is required in CO_2 modified SWC to ensure proper mixing of the mobile phase components, the application of radio waves (and the resultant decrease in the dielectric constant of water) might provide for better mixing of CO_2 and water below 75 °C, thereby allowing for separations in the mixed mobile phase system to be performed at even lower temperatures.

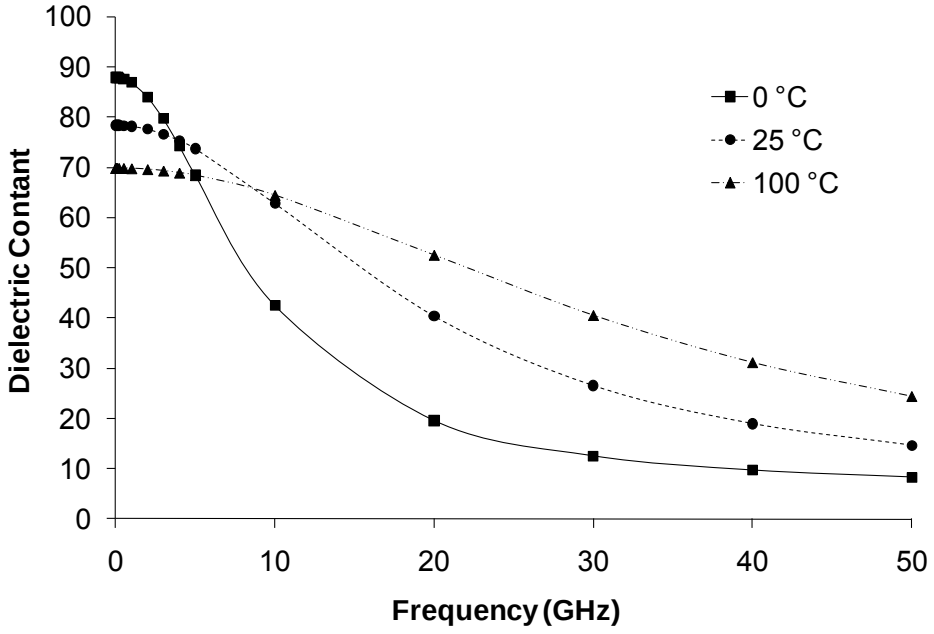


Figure 8-1: Relationship between the applied radio frequency and the dielectric constant of water at 0, 25, and 100 °C. (Figure compiled from reference 208)

8.2.2 CO_2 modified SWC

Chapter Three demonstrated the ability of CO_2 to significantly reduce the operating temperatures of SWC. In this regard, such a feature may potentially facilitate the use of stationary phases that have previously been precluded from use in SWC due to the high temperatures required such as bare silica and many monomeric-bonded silica phases.[103] A larger library of columns for use with the mixed mobile phase is beneficial since it allows for more options for method development. Therefore, further exploration of this system with other stationary phases would be advantageous in order to expand the applicability of this mode of chromatography.

In addition, since the temperatures employed in CO_2 modified SWC are significantly reduced when compared to that of conventional SWC, perhaps this technique would allow for the use of columns previously shown to induce analyte decomposition in SWC. For example, norpseudoephedrine has been shown stable on an ODS column but decomposed under elevated temperatures on a PBD-clad zirconia column.[125] Therefore, perhaps the reduced temperatures of CO_2 modified SWC would allow for the use of the PBD column for the analysis of norpseudoephedrine.

8.2.3 Pharmaceutical and other challenging separations

The stationary phases attempted in Chapter Four were too retentitive for the SWC elution of fluoxetine. Thus, perhaps a more appropriate choice of column would contain a highly crosslinked and end capped C8 or C4-bonded silica stationary phase. In this regard, heavily crosslinked silica phases help to minimise stationary phase erosion under SWC conditions[103] and end capping covers exposed silanol groups, which helps prevent hydrogen bonding between the analyte and the stationary phase. Also, since these columns have relatively low hydrophobicity[136], and they cannot form hydrogen bonds, π-π interactions, or dipole-dipole interactions with fluoxetine[137], they could potentially provide a better chance for fluoxetine elution in SWC.

The only successful SWC elution of fluoxetine was from a bare silica column and elevated temperatures resulted in a slightly improved peak shape. However, only 50 °C was attempted due to the rapid dissolution of silica in hot water. Conversely, an alternative stationary phase possibility is bare zirconia or titania, both of which do not readily dissolve under SWC conditions. In this regard, the use of these column options can allow for further increased temperatures while preserving the normal phase character

of the silica column. In this regard, perhaps these thermally stable stationary phase alternatives could provide a separation method for the SWC analysis of fluoxetine and other pharmaceutically relevant separations.

The CO_2 modified SWC system failed to elute fluoxetine, likely due to an on-column reaction between the secondary amine of fluoxetine and CO_2 itself. Alternatively, another challenging test analyte could be testosterone. Testosterone has been shown to be very highly retained in SWC, with one study only showing testosterone elution after more than two hours with the addition of 5 % methanol to the SWC mobile phase at 130 °C.[54] Since CO_2 acts in a similar fashion to organic co-solvents in reducing the net mobile phase polarity, testosterone could make an excellent candidate for the application of the CO_2 modified SWC system to probe its capabilities. As such, experiments should be performed in attempts to develop a separation method for the analysis of testosterone in the CO_2 modified SWC system.

8.2.4 Ultrasound in chromatography

The application of ultrasound has demonstrated a reduction in the observed peak splitting of a test analyte in the CO_2 modified SWC system. However, it is likely that the source of ultrasound used in these experiments was not strong enough to promote complete mixing of the mobile phase components under the conditions employed. This is further supported since other studies have found that a minimum of 0.5 W/cm^3 of ultrasonic energy was required for effective emulsification of water and CO_2.[151-153] Conversely, the ultrasonic bath employed in Chapter Five was only able to provide about 0.024 W/cm^3 of ultrasonic energy. Therefore, it is likely that a stronger source of ultrasound could promote much better mixing of the mobile phase components. This, in

turn, would possibly enable separations to be performed at lower temperatures without the appearance of peak splitting, thereby expanding the range of applicable analytes to this mode of chromatography. In this regard, future experiments should be performed with much more powerful sources of ultrasound to re-evaluate the effect ultrasound may have on the CO_2 modified SWC system.

8.2.5 Water stationary phase

Chapter Six describes a need for the further elucidation of the retention mechanism present while separating compounds on a water stationary phase. A potentially similar separation technique is hydrophilic interaction liquid chromatography (HILIC)[209], which employs a water-coated conventional HPLC stationary phase and a relatively non-polar mobile phase. The retention mechanism of this relatively more mature technique has been explored to a much greater extent in the literature.[210] Therefore, since the water stationary phase system employs a similar stationary phase to HILIC, perhaps comparative separations could confirm if the water stationary phase system also employs similar retention mechanisms. For example, if the selectivity and retention characteristics of test analytes are similar between these two systems, perhaps then they also share similar retention mechanisms. In this regard, comparative separations with HILIC may help explain the curious retention characteristics of the water stationary phase system (e.g. for tocopherol).

Further, since these two separation techniques may share similar retention mechanisms, applications of the water stationary phase system could potentially be derived from HILIC. For example, HILIC has been successfully applied to proteins,[211] pharmaceuticals,[212] and a wide range of other analytes including amino acids,

carbohydrates, glycols, pyrimidines, purines, glycans, alkaloids, folate species, antibiotics, and anti-cancer drugs.[210] If the retention mechanisms present in the water stationary phase are indeed similar to HILIC, it likely follows that the water stationary phase should be able to separate similar compounds. Therefore, these classes of compounds would make a logical next step for exploration into the application of the water stationary phase system. However, this extension hinges on the ability of the CO_2 mobile phase to elute them.

Chapter Six also concluded that the differences in analyte retention through a stainless steel tube and a fused silica tube could not be explained with only surface-water contact angle measurements. It was postulated that surface roughness or a heterogeneous surface of the stainless steel alloy could contribute to this difference. Therefore, investigations into the effect that the surface properties of stainless steel have on separations performed over a water stationary phase should be pursued. As such, to investigate the effect of the surface roughness of stainless steel, comparative separations could be performed in drawn-over-mandrel tubing and conventional tubing. Drawn-over-mandrel tubing is a cold-drawn, welded tube which provides for an excellent surface finish as compared to conventional, hot-extruded tubing. As such, differences in retention could help explore the effect surface roughness may have on separations and support the hypothesis that the relatively smoother, fused silica surface may be hampering the formation of the water stationary phase.

Further, to explore the effect of the surface heterogeneity of stainless steel, comparative separations could be performed on passivated and intentionally corroded tubing. Passivation promotes a uniform layer of chromium oxides on the surface of the

steel,[213] while exposure to chloride removes the layer of chromium oxide[214], allowing for further attack in an acidic environment.[215] Therefore, a uniform surface layer could be compared to an internationally corroded, heterogeneous surface and differences in retention could help explain the nature of the stainless steel tubing relative to its ability to support a water stationary phase.

8.2.6 Modified water stationary phase

The addition of salts to the mobile phase has been shown to alter the retention of weak acids in HPLC.[206] In Chapter Seven, attempts to emulate this work were performed by adding salts to the water stationary phase. One such attempt was performed to alter the retention of DBS by adding ionic compounds to the stationary phase. However, in this experiment, no change in DBS retention was observed upon the addition of salts to the stationary phase. In this regard, perhaps DBS was too strong of an acid for an effect to be noticed. Therefore, perhaps a less acidic analyte such as a carboxylic acid may make for a more appropriate test analyte to probe the effect of salts in the water stationary phase. In addition, other stationary phase additives that could also be evaluated include mercury and silver salts which have been shown to alter the retention of olefins and aromatics, respectively,[193] or perhaps chiral salts which could potentially affect the selectivity of chiral compounds.

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