



SUPERCritical FLUID CHROMATOGRAPHY

Primer



Agilent Technologies

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Primer

Terry A. Berger



Agilent Technologies

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FOREWORD

The author, Terry Berger has a unique, convoluted history with what we now call supercritical fluid chromatography (SFC), and is uniquely qualified to write this primer. Many of the early interactions were incidental and unfocused, but later meaningful. He brings nearly 40 years of relationships with people associated with SFC, along with over 35 years of direct experience. SFC had first been proposed nearly 60 years ago, in 1958, by Jim Lovelock, who later became a personal friend of the author through the 1980s and 1990s with numerous discussions about SFC.

Ernst Klesper demonstrated SFC experimentally in 1962 when he separated metal porphorins with dense chlorofluorocarbons as mobile phase. The author later interacted with Professor Klesper on numerous occasions, through the late 1970s to mid-1980s in both the USA and Germany, and again had extensive discussions about SFC.

Through the 1960s and 1970s, the technique largely languished with, at most, a dozen groups worldwide making a total of a few sporadic scientific contributions per year. Applications tended to be extensions of gas chromatography (GC) to higher molecular weight, higher boiling, relatively nonpolar solutes.

In the early 1970s, the author was a graduate student at Purdue University in Indiana. The adjacent laboratory was that of L.B. "Buck" Rodgers, a highly respected chromatographer at the time. After attending a scientific symposium in 1971, he came back determined to explore this new form of chromatography. Controlling the pressure of the mobile phase was the primary control variable, and pressure programming from low to high pressures was then the norm. All equipment in this period was homemade, with poor instrumental control. The physical and chemical characteristics of the mobile phases were primitively understood, at best. Rodgers rescued ultrahigh pressure hydrogenation pumps from storage, built an oven roughly 4-feet long that looked like a small coffin using 2-inch thick household foam insulation. Columns were 30 inches long or longer, and packed with irregular particles with a wide particle-size distribution. The author watched the progression of these developments and recalls that, at the time, being skeptical that this odd form of chromatography was useful. Rodgers results suggested there was a serious problem with density gradients along the length of

the column. This, and later work by Milos Novotny, seemed to indicate that the technique was unlikely to produce results as good as the current high-performance liquid chromatography (HPLC). This tended to suppress further work on packed-column SFC.

The author was trained in HPLC at Imperial College in London where he developed an amperometric electrochemical detector for HPLC, in the early 1970s. After subsequently teaching for a year in Brazil, he worked on a number of projects for instrumentation for spacecraft in Cleveland, Ohio.

The author was then hired by Hewlett-Packard in 1979, and joined the research group at the Avondale Division. Dennis Gere, Henk Lauer, Doug McManigill, and Harry Weaver, all scientists or engineers at Hewlett-Packard Labs or the Avondale Division, presented a series of talks on *dense gas chromatography* at that year's Pittsburg Conference (where the author was actually hired). Some of these talks were later published in such journals as *Analytical Chemistry*, and *Science*, as well as related application notes. These works resulted in a customer demand that led to the creation of a commercial kit that converted a Hewlett-Packard model HP 1084 HPLC instrument into an SFC system. Thus, commercial SFC was born.

This version of SFC used a mechanical backpressure regulator, a binary pump and composition programming, much like HPLC. Flow, composition, column temperature, and column outlet pressure were all independently controlled. Unlike previously home-built instruments, there was no pressure programming. Columns were more modern with much of the work done on spherical silica as small as 3 μm . The author's group produced the design for this kit.

About the same time that a capillary version of SFC, using pressure programming of pure CO_2 , was introduced, Hewlett-Packard made the HP 1084 instrument obsolete, replacing it with the SFC-incompatible HP 1090 HPLC instrument. Milos Novotny and Milton Lee were the drivers behind the capillary SFC development. This author's research group at Hewlett-Packard had just invented fused-silica capillary columns, capable of withstanding the high pressures (400 to 600 bar) necessary for capillary SFC. Milton Lee was an academic leader in the new field of bonding the stationary phase to capillary columns.

Packed-column SFC largely disappeared, due to lack of commercially available instrumentation, and capillary SFC was seen as the wave of the future. At this time, the physical chemistry of the fluids and their interaction with the stationary phase were largely unknown. Many controversies erupted pitting the few remaining packed column users against the capillary proponents. The author became the senior research scientist working with SFC at Hewlett-Packard in 1985, and was assigned to unravel these controversies. In the process, he visited every European user (about 16) for extensive discussions about the controversies and potential.

At the time, it was widely believed that density was the primary control variable, and that changing the mobile phase density had the biggest effect on retention. It was thought that dense CO_2 was as polar as isopropanol, due to an unfortunate misunderstanding by Giddings¹. If it were true, adding an alcohol modifier could change density significantly, but only insignificantly change solvent strength. This misinterpretation was not challenged for decades. At the time, there were *no* direct density measurements of methanol/ CO_2 (or any other) mixtures used in SFC. Equations of state for binary mixtures were inaccurate. The author subsequently borrowed a densitometer from the University of Delaware and made some of the first density measurements of methanol/ CO_2 in the literature. He then measured the effect of methanol concentration on retention at constant density, showing that the modifier was indeed responsible for dramatically increasing the elutropic strength of the mobile phase.

Not long afterwards, solvatochromic dyes were shown to offer a simple way to measure the strength and polarity of the solvation sphere surrounding the dye molecules in HPLC². The author then applied the same principle to SFC⁴⁵. The results indicated that the solvent strength of binary fluids was highly nonlinear, with the first small additions having an inordinate effect. CO_2 was shown to be similar to pentane in polarity and solvent strength.

With density and solvatochromic dye measurements, the Giddings' elutropic series¹ was conclusively revised, although it still has followers. The result is that changing modifier concentration was shown to have a much larger impact on retention than pressure, temperature, and density, at least for modified fluids.

It was widely demonstrated during the late 1980s that many polar solutes such as organic acids and bases eluted with poor peak shapes or did not elute at all when using binary fluids consisting of CO_2 modified with an alcohol such as methanol or ethanol. It was generally thought that this indicated that the stationary phase was too active or contained

active sites that interacted too strongly with these polar solutes. The proposed solution was to use less-polar stationary phases, with further deactivation. This approach simply did not work. Polar molecules typically went unretained and often still tailed badly. The author introduced the use of a polar additive such as a strong acid or base, or both, mixed with the organic modifier, along with the use of more polar columns. This approach was shown to dramatically improve peak shapes. The use of such additives opened up the possibility for what is modern SFC, with the elution and separation of polar solutes that now includes peptides up to 40'mers.

Going back to at least "Buck" Rodgers papers³ there was a perception that pressure drops across the column resulted in density gradients, causing serious efficiency losses. This was reinforced by Novotny in the mid-1970s⁴. By the mid-1980s there were multiple competing theories that at least one of which⁵ suggested that packed-column SFC was incapable of generating more than about 20,000 plates, due to the density gradients accompanying the pressure drops across the column. The author connected eleven 20-cm long columns with 5- μ m packing in series, and demonstrated 220,000 plates with pressure drops of up to 250 bar, conclusively refuting most of the arguments. The effect of density gradients continues to be an area of interest and the topic of numerous recent publications.

The author convinced Jon Parcher at the University of Mississippi to perform tracer-pulse MS experiments to measure the adsorption of mobile-phase components onto the stationary phase. The results indicated that the extensive adsorbed films had a dramatic effect on the nature of the stationary phase, and subsequently, on the chromatography.

With most of the misconceptions disproved, the author convinced Hewlett-Packard management to fund a next-generation SFC project. The resulting product was released in 1992, and was capable of both capillary and packed-column performance. This instrument, the HP G1205A, could simultaneously and independently control flow, composition, pressure, and temperature. It could also perform density programming using an internal equation of state.

With the improved understanding of the mobile-phase characteristics by the early to mid-1990s, it was widely recognized that capillary SFC had been greatly oversold, particularly for polar solutes. This recognition was largely due to the revision of Giddings' elutropic series. Sadly this resulted in the death of capillary SFC, even though it had demonstrated numerous advantages over other techniques with less polar solutes.

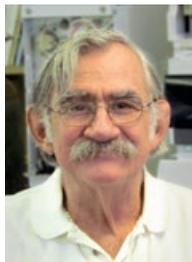
In 1995, the author had the opportunity to buy the SFC business from Hewlett-Packard, and, along with another former employee and an outside investor, created Berger Instruments (BI). BI refocused the design to primarily perform packed-column SFC, and reduce cost. BI introduced the first chiral method development system, the first successful semipreparative instruments for both multigram (mostly chiral) and high-throughput library purification, the first commercial SFC-MS system, the first successful gas-delivery system, and a number of other minor products. The company was sold to Mettler Toledo in late 2000, where the author served as Chief Technical Officer until 2003.

In 2008 the author started Aurora SFC Systems, which produced a module that converted a more or less standard HPLC instrument into a state-of-the-art SFC system. Aurora was sold to Agilent Technologies in 2012, and the upgraded module is now at the heart of the Agilent analytical SFC system. The author's R&D group has been awarded two different R&D 100 awards, one at BI and one at Aurora, each for designing one of the 100 top inventions in 2004 and 2008.

The author currently has an SFC consulting business (SFC Solutions, Inc.), with a state-of-the-art SFC laboratory. Freed from the necessity of running a business, he is actively producing research and is publishing original research on instrumentation, columns, and applications, including this primer.

The author's long-term, deep understanding of the physical chemistry of the fluids, applications and instrumentation of SFC, is unique in the industry. He has seen many fads come and go, and many negative theories fade away. He is extremely optimistic about the future of SFC. It is hoped that this deep understanding can be transferred to the reader of this primer in such a way that they can enjoy great success in implementing SFC in their laboratory.

ABOUT THE AUTHOR



Terry A. Berger

PhD, DIC

President, SFC Solutions, Inc.

Terry A. Berger received his PhD in analytical chemistry from Imperial College of Science and Technology, University of London, UK, in 1976, following graduate work at Florida, Wisconsin and Perdue. Dr. Berger is considered by many to be the father of modern supercritical fluid chromatography (SFC). In 2004, Dr. Berger was awarded the Martin Gold Medal by the Chromatographic Society of Great Britain, one of the premier awards in chromatography.

Starting in 1985, at Hewlett-Packard (now Agilent Technologies), he spent more than a decade systematically undoing many of the misconceptions about packed column SFC that were then prevalent. In the process, he untangled the effects of density and solvent strength, which had been major points of contention. He showed that, contrary to several proposed theories, large pressure drops did *not* result in serious losses in efficiency (a point still of active interest). He introduced the use of additives and systematically studied their effect on peak shape and retention. He demonstrated the first separations of broad classes of compounds and, against the common perception, showed that packed-column SFC was broadly applicable to many more polar solutes than previously thought possible. Examples included small drug-like molecules such as primary amines.

Dr. Berger's book *Packed Column SFC*, published by the Royal Society of Chemistry in 1995, fundamentally changed the perception of SFC in separation science, making modern SFC more similar to HPLC than GC. This book has remained the primary source for understanding of the fundamentals of the technique, up to the publication of this primer, which is intended to supersede it. Other books on SFC, published before this primer, are compendia by numerous authors of specific topics.

Dr. Berger has also published about 75 peer-reviewed scientific papers, 7 book chapters, 13 encyclopedia and reference work entries, and dozens of application notes. He has taught more than 20 short courses on SFC, given hundreds of oral presentations and posters, and has received or applied for about 25 patents in GC, SFC, and HPLC.

In 1995, he formed Berger Instruments, which introduced the first SFC chiral method systems, the first successful semipreparative SFC system for both library purification and multigram separations, the first commercial SFC-MS, as well as the first gas-delivery systems. Later he founded Aurora SFC, which built a module to convert analytical-scale HPLC instruments into SFC systems. This module became the basis for the Agilent analytical SFC system and the Agilent analytical SFC/UHPLC hybrid system.

His technical teams have won two R&D 100 Awards over the last 12 years. They have also won a NICE III environmental award from the Department of Energy. Dr Berger continues to be an editor and reviewer for chromatographic publications and societies.

INTRODUCTION

This primer is intended to supersede the author's book *Packed Column SFC*, which was first published in 1995 by the Royal Society of Chemistry. This original work fundamentally changed the perception of SFC in separation science making modern SFC more similar to HPLC than GC. This perception is continued and expanded upon in this primer.

While the first chapter describes some of the basic principles of SFC, it also discusses the rationale, motivation and benefits of deploying the technique in the modern analytical laboratory. The following chapters provide a more detailed review of the mobile and stationary phases as well as the effects of these phases on the important chromatographic characteristics of retention and selectivity.

A primer on separation science would not be complete without a thorough discussion on the practical implementation of the technique of interest, including the fields of application. This is covered in chapters on method development, achiral and chiral separation, and on quantification.

Finally, instrumental considerations are discussed, including detailed explanations of critical aspects of mobile-phase delivery, sample injection, and detection.

Symbols

A	contribution of eddy diffusion in van Deemter equation
B	contribution of axial diffusion in van Deemter equation
C	contribution of radial diffusion in van Deemter equation
D	solute binary diffusion coefficient in mobile phase
d_p	particle diameter
H	plate height
k	partition ratio
μ	linear velocity of mobile phase
P	partition coefficient
P'	elution strength (according to Snyder)
ΔP	pressure drop across separation column

Abbreviations

ACN	acetonitrile
BPR	backpressure regulator
CZE	capillary zone electrophoresis
EtOH	ethanol
FID	flame ionization detector
GC	gas chromatography
HPLC	high performance liquid chromatography
id	inside diameter
IPA	isopropanol (isopropyl alcohol)
IPAm	isopropylamine
MeOH	methanol
MS	mass spectrometer
RI	refractive index
RSD	relative standard deviation
SFC	supercritical fluid chromatography
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
UHPLC	ultrahigh pressure liquid chromatography
UV	ultraviolet

1.1 What is SFC?

As supercritical fluid chromatography (SFC) is a separation technique, which uses instrumentation that is almost identical to that used in high performance liquid chromatography (HPLC). Complex mixtures can be separated and the amount, and sometimes the identity, of the individual components in the mixture can be determined. A solution of the sample is injected into a high-pressure flow stream that sweeps the sample into a tube or column filled with fine particles. The individual components in the sample interact differently with the surface of the particles, and are separated in time and space as they pass through the column. The components emerge from the column at different times, as Gaussian or pseudo-Gaussian peaks, and pass through a detector.

The most significant difference from HPLC is the replacement of most of the liquid mobile phase with a dense compressed gas, almost always carbon dioxide (CO_2). At high pressures such as greater than 80 bar, CO_2 acts as a solvent. Because it is a compressed gas, a backpressure regulator is required on the system outlet to ensure the mobile phase remains a single dense phase throughout the chromatograph. This, in turn, requires some detectors, such as an ultraviolet (UV) detector, to be operated at elevated pressures.

CO_2 is a highly nonpolar solvent, similar to a hydrocarbon but in a different solvent family. Consequently, for more polar solutes, an organic modifier, sometimes called a cosolvent and most often an alcohol, is added to the mobile phase. Gradient elution from low to high modifier concentration is the norm. Peaks elute from lower to higher polarity.

For many highly polar solutes, the interactions with the stationary phase are too intense, and the solutes often fail to elute, or elute with poor peak shapes. This problem can usually be solved by including a highly polar additive in the mobile phase such as a strong acid or base dissolved in the modifier.

SFC is usually a *normal phase* technique because composition is programmed from low to high polarity. However, SFC has significant advantages compared to normal phase HPLC. Equilibration is extremely fast, reproducibility is excellent, and even aqueous-based samples can be injected.

For polar solutes, polar stationary phases are used. Classical polar phases included bare silica, cyano, diol and amino. In the last few years, a number of new stationary phases have been developed specifically for SFC. These phases include several ethylpyridines and a number of proprietary phases. For low polarity solutes, reversed phase columns such as C18, C8, C4, and methyl are sometimes used.

Over the last few years, the use of sub-2- μm particles has become fairly common. However, the dominant use of SFC in the past several decades has been in chiral separations^{6,7}. The same columns used in HPLC are used in SFC. Although the most effective older chiral columns were coated and not bonded, the newer bonded phases have not yet displaced the coated columns due to superior selectivity and less complex optimization.

SFC is also useful for the separation of much less polar compounds such as many natural products, including fat soluble vitamins, carotenoids, and lipids. With such samples the stationary phase is usually C18.

1.2 Why deploy SFC?

1.2.1 Faster analysis times

The van Deemter (Knox) equation describes the kinetic performance of a chromatographic column. In its simplest form, this equation comprises three terms, each describing a different form of diffusion, see Equation 1.1.

$$H = A^{0.33} + \frac{B D_{1,2}}{\mu} + \frac{C d_p^2 \mu}{D_{1,2}}$$

Equation 1.1 The van Deemter (Knox) equation.

The terms B and C indicate the contribution of axial and radial diffusion, and contain the ratio of the solute binary diffusion coefficient in the mobile phase, $D_{1,2}$, to the mobile phase linear velocity, μ . The term B indicates that higher diffusion coefficients result in higher optimum linear velocities. In term C , a higher diffusion coefficient results in less loss of efficiency at higher flow rates.

Diffusion coefficients in pure CO₂ are in the order of 10 to 15-times faster than in water or aqueous mixtures. For example, the diffusion coefficient of benzoic acid in water is $1.0 \times 10^{-5} \text{ cm}^2\text{s}^{-1}$ at 20 °C⁸. In 100 % CO₂, the diffusion coefficient of benzoic acid is $16 \times 10^{-5} \text{ cm}^2\text{s}^{-1}$ at 100 bar and 40 °C, dropping to about $9.5 \times 10^{-5} \text{ cm}^2\text{s}^{-1}$ at 300 bar⁹. Similarly, several slightly larger dimethyl anilines exhibited diffusion coefficients between 7 and $12.5 \times 10^{-5} \text{ cm}^2\text{s}^{-1}$ between 150 and 350 bar, and 40 to 60 °C¹⁰.

The intermolecular interactions between CO₂ molecules are weak. Consequently, it is a gas at room temperature and atmospheric pressure. When such molecules are forced close together (compressed), the resulting dense fluid acts as a solvent. Nevertheless, the intermolecular forces are still weak even at the higher density. Consequently, other compounds dissolved in the CO₂ diffuse rapidly through it.

In recent years, SFC has seldom been performed using pure CO₂. The addition of a polar modifier, for example, an alcohol such as methanol, ethanol or isopropanol, decreases diffusion coefficients significantly. The addition of only 5.5 % methanol decreases diffusion coefficients by nearly half¹¹. Higher concentrations have an even larger effect. For example, malvidin-3,5-diglucoside¹² has a molecular weight of 670, making it fairly large, with a number of polar functional groups, compared to many small drug-like molecules. With 20 % methanol in CO₂, and at 200 bar, the diffusion coefficient was still 4.7 to 5.1-times higher than the diffusion coefficient in pure methanol at atmospheric pressure and at 40 to 60 °C, as shown in Figure 1.1. It has been shown that polar solutes are surrounded by a *cluster* of the polar modifier, and this larger solvated entity diffuses more slowly^{11,13}, due to the increased cross-sectional area of the cluster.

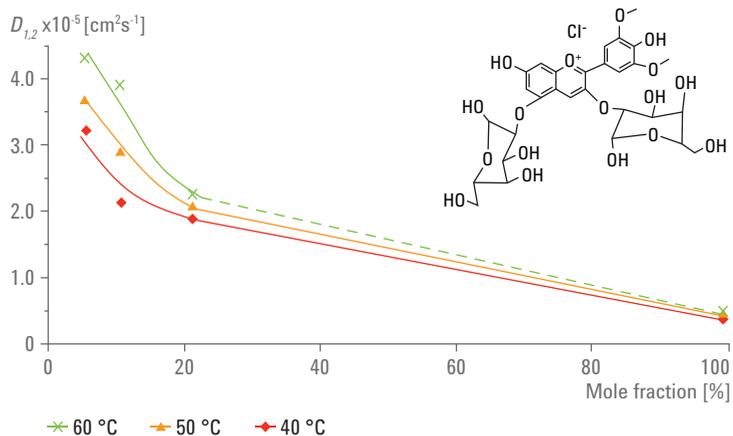


Figure 1.1 Diffusion coefficient of malvidin-3,5-diglucoside, which has a molecular weight of 670, in CO_2 /MeOH mixtures. The CO_2 /MeOH data was collected at 200 bar. The pure MeOH data was collected at atmospheric pressure. At 20 % modifier, the diffusion coefficients were 5.08, 4.50, and 4.70-times higher than in pure MeOH at the same temperatures.

Since SFC is usually performed with between 5 and 50 % modifier, it is typically stated that SFC, with modified mobile phases, is three to five-times faster than HPLC, with the same chromatographic efficiency, on the same sized particles. This is still true when using columns packed with sub-2- μm particles. This means that run times are one-third to one-fifth as long and throughput is three to five-times greater than in HPLC (or UHPLC), on the same sized column. Re-equilibration is fast, resulting in short cycle times for gradient analysis.

Smaller molecules have higher diffusion coefficients. Unfortunately, there is little data in the literature about the diffusion coefficients of small molecules in modified CO_2 . Most of the values in the literature are presented in Table 1.1.

Solute	Temperature	Pressure	$D_{1,2}$ [cm^2s^{-1}]		
			5 % MeOH	5.5 % MeOH	10 % MeOH
2-nitroanisol	40 °C	200 bar	7.8×10^{-5}		7.22×10^{-5}
		300 bar	6.85×10^{-5}		6.25×10^{-5}
	50 °C	200 bar	8.36×10^{-5}		7.56×10^{-5}
		300 bar	7.52×10^{-5}		6.76×10^{-5}
Dichlorobenzene	40 °C	200 bar	9.36×10^{-5}		8.47×10^{-5}
		300 bar	7.98×10^{-5}		7.6×10^{-5}
t-butyl benzene	50 °C	200 bar	8.33×10^{-5}		7.57×10^{-5}
Acridine	55 °C	173 bar		7.78×10^{-5}	
Benzoic acid	55 °C	173 bar		6.69×10^{-5}	
Phenanthrene	55 °C	173 bar		10.0×10^{-5}	

Table 1.1 Diffusion coefficients of a few small molecules with small additions of methanol to CO_2 ^{10, 11}.

1.2.2 Lower pressure drops

The same low intermolecular interactions that result in higher diffusion coefficients also result in low viscosity of both pure and modified CO_2 . If we ignore 100 % CO_2 for the moment and concentrate on modified CO_2 , Figure 1.2 shows literature¹⁴ viscosity values for mixtures of water/methanol, at 40 and 60 °C, and water/acetonitrile at 60 °C. These HPLC-like conditions are compared to values for CO_2 /methanol mixtures estimated from measured pressure drops (ΔP) across an SFC column, at 50 °C, extrapolated to 100 % methanol (dashed line). The aqueous-based measurements were obtained at constant pressure. The CO_2 /methanol measurements were obtained from measurements between about 200 and 400 bar at constant flow.

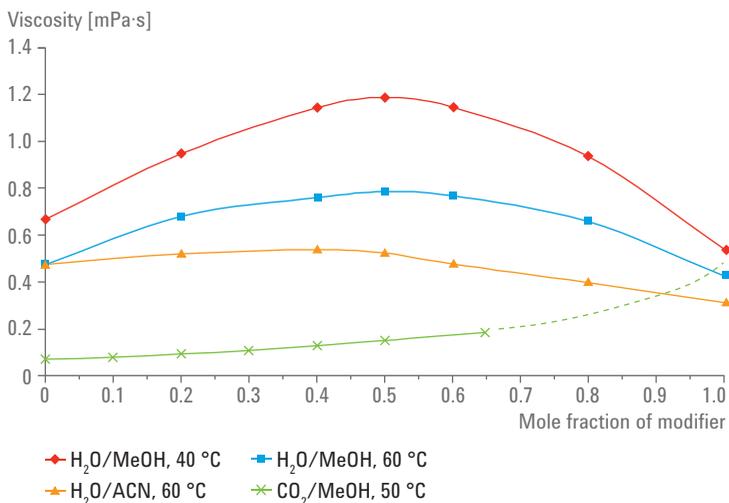


Figure 1.2 Viscosity of H₂O/MeOH, H₂O/ACN, and CO₂/MeOH mixtures as a function of the mole fraction of the modifier. The pressure drop across the column is directly related to the viscosity.

In the late 1990s there was a revival of the use of sub-2- μm particles, but these were mostly pellicular and were often packed in capillaries. Small diameter, superficially porous particles then appeared. In 2003, Agilent introduced the first totally porous, sub-2- μm particles. Such particles offered the possibility of increasing analysis speed nine- or tenfold, compared to 5- μm particles, with the same efficiency, according to the van Deemter equation.

These smaller particles generated much higher pressure drops (ΔP) than the older, larger particles, since ΔP is proportional to d_p^2 (at constant efficiency). The use of sub-2-micron particles in HPLC sometimes requires pumps capable of greater than 1000 bar. In SFC, the viscosity of CO₂/modifier mixtures is dramatically lower than the aqueous-based fluids, as shown at the bottom of Figure 1.2. Subsequently, ΔP values are much lower than in HPLC, even at higher flow rates. It is actually unusual to exceed 400 bar in SFC, even using 1.8- μm particles in columns of 100 mm in length. The ΔP of several columns packed with 3.5 and 1.8- μm particles was plotted against percent of modifier at constant outlet pressure, and various flow rates. The results are presented in Figure 1.3 and Figure 1.4, respectively.

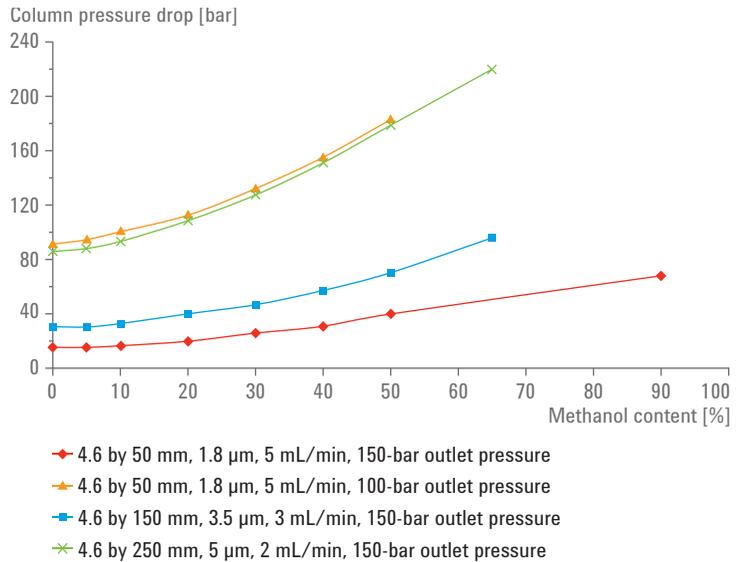


Figure 1.3 Pressure drop across several ZORBAX RX-Sil columns at 50 °C, with various concentrations of MeOH in CO₂, with the ΔP due to tubing subtracted.

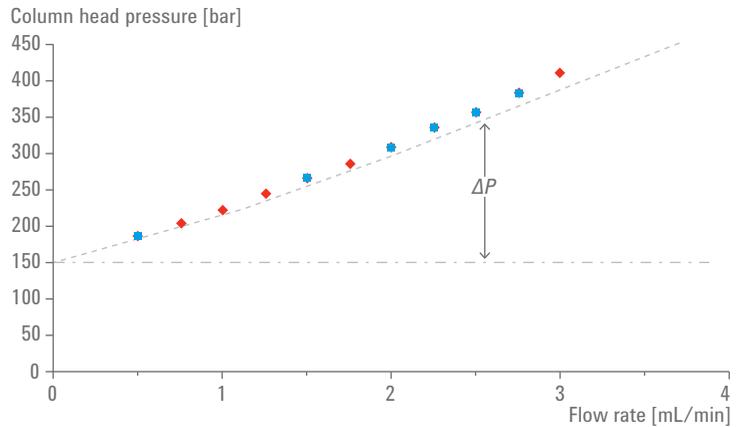


Figure 1.4 Column head pressure using a 3 by 100 mm, 1.8 μm ZORBAX RX-Sil column, with 22.5 % methanol in CO₂, 150-bar outlet pressure, 50 °C. The optimum flow rate is approximately 1.8 mL/min.

Simply stated, modern HPLC with sub-2- μm particles is often performed at elevated temperatures from 40 up to 70 $^{\circ}\text{C}$, in order to decrease the viscosity and pressure drop. SFC is generally performed between 40 and 60 $^{\circ}\text{C}$, but for completely different reasons. It is a general rule of thumb that ΔP in SFC is one third to one fifth of the ΔP in HPLC at three to five-times higher flow rates. There is no real requirement for ultrahigh pressure pumping in SFC, unless you use long columns packed with sub-2- μm particles or are operating at exceedingly high flow rates, either of which can be desirable.

1.2.3 Orthogonal to reversed phase HPLC

A minor component is often chemically similar to, and elutes near or even under a major component using any one technique. Several methods with different selectivity may be required for a single sample. However, two different reversed phase methods are often used, both using a C18 stationary phase to try to resolve such coelution. An orthogonal technique, like normal phase HPLC or SFC provides a superior alternative for significantly reducing the risk of missing such coeluting compounds. SFC should be preferred since it is faster, uses less hazardous modifiers, and generates much less toxic waste.

The retention order of any group of peaks in SFC is vaguely opposite to the retention order in reversed phase HPLC. A typical example¹⁵ is presented in Figure 1.5. The solutes represent a diverse group of functionalities, including sulfonamides, corticosteroids and xanthenes. Such major changes in selectivity should be highly desirable. Two separation mechanisms with different chemical interactions have positive consequences. Major components often have a *tail* where the signal does not return to the baseline in a timely manner. If the minor component coelutes, or elutes on the tail of the major component, it may be difficult, or impossible, to quantify the minor component with any precision. If, however, the minor component elutes before the major component, where the baseline is flat, it might be more easily quantified with higher precision and accuracy.

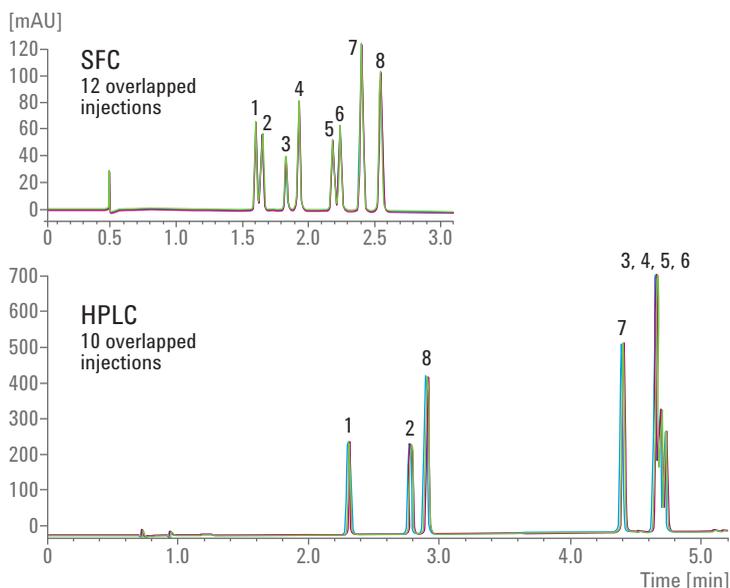


Figure 1.5 SFC is orthogonal to reversed phase HPLC. Comparison of SFC and HPLC chromatograms for a mixture of: 1. caffeine, 2. theophylline, 3. cortisone, 4. prednisone, 5. hydrocortisone, 6. prednisilone, 7. sulfamerazine, 8. sulfaquinolaxaline. SFC Conditions: 4 mL/min of 5 to 25 % methanol in CO₂ in 3 minutes. 150-bar outlet pressure with a 4.6 by 150 mm, 5 μ m, RX-SIL column. HPLC conditions: 1.5 mL/min of 10 to 90 % methanol in water in 4.5 minutes, 40 °C, on a 4.6 by 150 mm, 2.7 μ m, Poroshell C18 column¹⁹.

The Agilent analytical SFC/UHPLC hybrid system provides for rapid switching back and forth between reversed phase HPLC and SFC in minutes. Sequences can be programmed to perform such comparisons automatically. This facilitates sequential running of two methods with the same sample, using dramatically different selectivity, one reversed phase the other normal phase, in a short period of time. The chromatograms presented in Figure 1.5 are just such an example. Six SFC runs were performed followed by six reversed phase HPLC runs, followed by four SFC runs, then four reversed phase HPLC runs and finally two SFC runs. All the SFC and reversed phase HPLC runs were then superimposed.

1.2.4

Separation of chiral and other isomers

Reversed phase HPLC relies mostly on differences in hydrophobic interactions to separate compounds. Normal phase techniques rely mostly on polar-polar interactions, and can better differentiate subtle differences in shape, particularly around polar functional groups. As a consequence, a typical application area of normal phase HPLC is in the separation of isomers, where the separation is based on shape of the molecule.

Most drugs are chiral, meaning they have pairs of isomers that are mirror images of each other called enantiomers. Mixtures containing equal concentrations of the enantiomers are called racemic. Older synthetic approaches almost always made racemic mixtures. However, each enantiomer often has unique biological activity. Consequently, the US Food and Drug Administration and many other regulatory agencies require full testing of each pure enantiomer. It has also been possible to repatent a drug as a pure enantiomer since the pure enantiomer is often faster acting and less toxic than the older racemic form. Because of these factors, the demand for chromatographic separation of enantiomers has increased.

Over the last 20 years SFC has been proven to be superior to normal phase HPLC for the separation of enantiomers and other isomers. In fact, many larger pharmaceutical companies have focused on SFC for these applications. For example, Craig White⁶, who, at the time ran an analytical and semipreparative purification service laboratory, analyzed all chiral samples submitted to his laboratory for one year, which amounted to hundreds of samples. In 96 % of the separations, SFC was found superior, in terms of speed and resolution. In fact, SFC was subsequently designated as the primary technique for both analytical, and semipreparative chiral separations and HPLC was only used to evaluate the SFC problem samples.

Similarly, Mohammed Maftouh⁷, evaluated 500 proprietary drugs and had a 95 % success rate by SFC using only four older (coated, not bonded) chiral stationary phases. With a set of 98 marketed drugs they had a 98 % success rate with the same system. Since 2006 they have been using SFC as the primary screen, with capillary zone electrophoresis (CZE) as their back-up. Both Pfizer and Merck have also published similar but slightly less definitive statements about the value of SFC, for chiral separations.

1.2.5 Higher efficiency

The low ΔP values in SFC make it easy to generate high efficiency. Columns can be, and often are, linked in series. Either the same or different stationary phases have been coupled in this manner. Both chiral and achiral columns have been coupled. In the earliest example¹⁵, eleven columns, 200-mm long each, were coupled to produce a column 2.2-m long (5- μm particles) producing over 220,000 plates, in a 400-bar system. In another example, up to five different chiral columns have been coupled to make a pseudo-universal phase¹⁶.

Alternately, long columns packed with sub-2- μm particles can be used to generate reasonably high efficiencies because the pressure drops are low. The Agilent analytical SFC system is capable of 600-bar operation, which would support such a column about 0.5-m long, theoretically producing up to 139,000 plates.

1.2.6

Lower operating costs

Most of the CO_2 used in SFC is food or beverage grade. Since it is meant for human consumption, its purity is regulated. Carbonated beverages are ubiquitous, the distribution infrastructure is in place, more or less, worldwide, and huge volumes are consumed. Consequently, CO_2 is inexpensive. In high-pressure (50 to 70 bar) steel cylinders (25 kg), liquid CO_2 can be as inexpensive as US\$ 1 per kilogram. Typical usage in SFC is 1 to 3 mL/min, meaning a cylinder lasts about 200 hours. CO_2 is also available in Dewar flasks and bulk tanks both of which operate cryogenically at -30 to -40 °C, and 20 to 30 bar. Dewar flasks generally hold about 150 kg, but are less convenient than either cylinders or bulk tanks. The CO_2 in Dewar flasks generally cost about the same as in steel cylinders, but last six times longer. Bulk tanks are usually installed only when the organization commits to large-scale use of SFC. The cost of the CO_2 from a bulk tank can drop an order of magnitude to US\$ 0.10 per liter. This compares favorably with the cost of heptane, which can exceed US\$ 70 per liter when purchased in cases of four 4-liter bottles.

Both Dewar flasks and bulk tanks require a booster pump or gas delivery system to increase the pressure from about 20 to above 70 bar in order for a reciprocating pump to be able to pump it. With bulk tanks, the CO_2 is generally plumbed to multiple locations within a facility.

In SFC, expensive, toxic acetonitrile is seldom used. Instead, an inexpensive alcohol is used. Further, typical operation starts at a low %B such as 5 % but seldom exceeds 40 %. Thus, most of the mobile phase is inexpensive CO_2 .

At the end of the chromatograph, the mobile phase decompresses and forms two phases, one gaseous, and the other a liquid. The gaseous CO_2 is vented, while the liquid modifier is collected in a trap. Since most of the mobile phase evaporates, the volume of liquid waste is greatly reduced. What waste that is generated is also less toxic.

Labor costs are one of the single largest expenses when operating a chromatograph. Since higher diffusion coefficients dictate higher flow rates and shorter run times in SFC compared to HPLC, on the same-sized column, a single operator produces three to five-times more work in the same time frame.

1.2.7 Environmentally friendly

Despite using CO₂ as the main fluid, SFC is considered environmentally friendly or *green* because the CO₂ is recycled from other industries. In HPLC, the mobile phase is often burned after use, generating *new* CO₂. Toxicity can be decreased by using small concentrations of alcohols instead of acetonitrile. Since the CO₂ evaporates at the end of the system, the volumes of toxic liquid waste requiring expensive disposal is greatly reduced.

1.3 What can SFC separate?

As a rule of thumb, any compound soluble in methanol or a less polar solvent, is a good candidate for separation by SFC¹⁷. Conversely, compounds that require a completely aqueous, buffered solution to dissolve are probably poor candidates. This should not be interpreted as SFC being incompatible with water in the mobile phase, aqueous samples, or many biological samples. There is a review of the use of water as part of the SFC mobile phase¹⁸.

Although SFC is not thought of as appropriate for large biomolecules such as proteins, peptides up to 40'mers have been eluted^{19–21}. Further, peptide isomers varying only in the position of one amino acid have been well separated in short times^{22,23}. This is an area of active research. If a peptide can be eluted, it is likely to have significantly enhanced chromatographic speed under the conditions used compared to HPLC.

In the early days of SFC most applications involved the separation of relatively nonpolar solutes, often homologous series, such as silicone oils, surfactants, waxes, lipids, and the like that were too high in molecular weight, or too thermally labile for high-temperature gas chromatography (GC). Most of this work was performed using pure CO₂, pressure programming, and a flame ionization detector (FID). This slowly started to change in the late 1980s, particularly after the first chiral SFC separations were published^{24–26}.

Over the last 15 years, SFC has been used largely in the pharmaceutical industry for the rapid elution of small drug-like molecules, particularly for chiral separations. With recent improvements in robustness and particularly in UV sensitivity, SFC has started to re-expand into a much wider range of applications.

The application areas of SFC are compared to the application areas of the various forms of liquid chromatography in Figure 1.6. As shown, SFC with various mobile phase combinations covers nearly the same application space as HPLC in its various forms. The only area not significantly covered is ion chromatography.

Where Does SFC Fit Relative to HPLC?

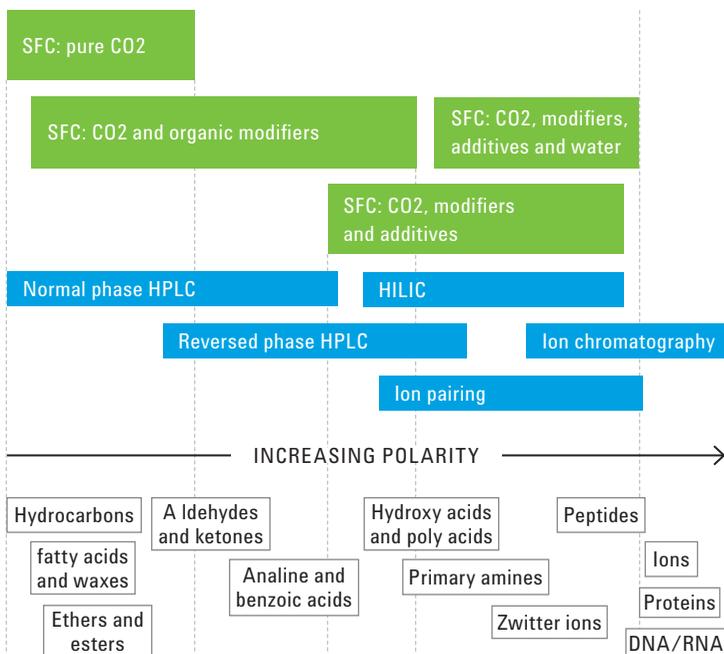


Figure 1.6 Comparison of various forms of SFC to those used in the subsets of HPLC, showing that SFC largely covers the same application space as HPLC.

1.3.1 Specific aspects of retention in SFC

Normal phase SFC is about polar-polar interactions between the solutes and a polar stationary phase. Steric hindrance is important in SFC. A polar functional group, largely surrounded by nonpolar moieties within the same molecule, tends to result in much less retention of the molecule on polar stationary phases, compared to a molecule with the polar functionality unhindered. For example, in the series mono-, di-, and tribenzylamine the basicity increases with the degree of substitution on the nitrogen²⁷. Thus, tribenzylamine is a stronger base and is more polar than dibenzylamine, which, in turn, is a stronger base and is more polar than monobenzylamine. In this series, the molecular weight also increases with the number of phenyl substitutions. Larger molecules tend to be more retained. If polarity and molecular weight were the determining factors, the elution order on a polar column ought to be benzylamine followed by dibenzylamine and then tribenzylamine. However, tribenzylamine is almost unretained, dibenzylamine elutes second, and monobenzylamine as the smallest and weakest base is the most retained. Clearly, each additional substitution of a phenyl group on the nitrogen progressively hinders the lone pair on the nitrogen, getting close to and interacting with the polar

stationary phase. For similar reasons, nitrogen atoms in rings tend to have weak interactions with polar stationary phases and seldom need an additive to achieve good peak shapes.

The choice of stationary phase can have a large impact on the speed of analysis. Some stationary phases are much more retentive than others. If two different stationary phases provide reasonable solutions to an analytical problem, the one using the lower modifier concentrations should generally be preferred since the lower the modifier concentration, the lower the viscosity and column pressure drop.

1.3.2 LogP

A widely used measure of lipophilicity in drug development is $\log P$, which is the logarithm of the partition coefficient (P) of the compound between octanol and water. Octanol is an alcohol, making the hydroxyl head moderately polar, while the C8 backbone is nonpolar. Octanol and water are not miscible and form two distinct phases when brought in contact with each other, giving the molecules of the compound of interest the *choice* of which phase to be in.

A positive $\log P$ means the drug partitions preferentially into the octanol. A negative $\log P$ means it preferentially partitions into the water. Lipinski created a set of rules he called the *rules-of-5* for effective drug development²⁹. One of the rules stated that to be an effective oral drug, the compounds $\log P$ should be between 0 and 5. With a $\log P$ of 2.5, more than 300 molecules would partition into the lower polarity octanol, for each molecule that partitions into the water.

The distribution of actual $\log P$ values of 5000 commercial drugs is plotted in Figure 1.7, and shows the largest drop between -1 and 6 , with an average of about 3 , just as Lipinski suggested. Solutes with a range of $\log P$ from less than -2 to greater than 7 have been eluted and separated using CO_2 -based mobile phases. Thus, SFC is ideal for the elution and separation of small drug-like molecules.

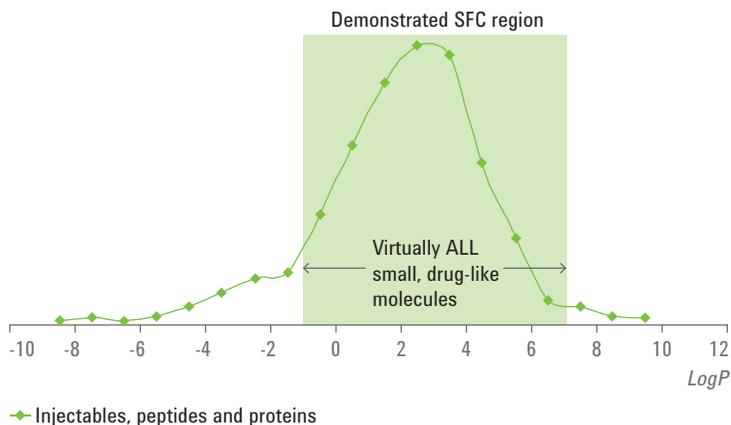


Figure 1.7 A plot of the log of the partition coefficient between octanol and water ($\log P$) of 5000 commercially available drugs, which shows that the vast majority is only moderately polar and fit well with SFC.

The rule of thumb suggesting SFC is not appropriate for compounds requiring an aqueous buffered environment has, in the past, been misinterpreted as meaning SFC was only appropriate for nonpolar solutes, which is untrue. Many organics are very poorly soluble in water ($\log P$ much greater than 5). This makes them poor candidates as drugs, since they would be permanently adsorbed into fatty tissues and would never interact with the appropriate biological receptors. A major effort in drug development is often to increase water solubility, to decrease the likelihood of the compound being stored in fatty tissues. This can also be misinterpreted to suggest that drug-like molecules must be highly soluble in water (and biological fluids). This is also not true. If compounds are too water-soluble, they would be poor drug candidates, since they would be quickly eliminated from the body. Increasing water solubility often means increasing from *insoluble* to only *slightly soluble*. Such compounds are likely to spend a significant amount of time in the cells, interacting with the appropriate receptors. Thus, such compounds are considered to be moderately fat loving, or lipophilic.

Another *rule-of-5* states that ideal oral drugs should have a molecular weight no greater than 500²⁸. In Figure 1.7, there are several drugs with $\log P$ of -2 and below. These are mostly proteins and large peptides, which are too large to pass through the gut intact, and are destroyed making them inappropriate for oral administration. Instead, they are injectable. Such compounds are mostly not amenable to separation by SFC.

1.4 What's in a name?

Too much is usually read into the prefix *super* in supercritical because it seems to suggest that something special happens when you *cross the border*. However, *super* simply means *above*. Similarly, *critical* is also ominous sounding and is often misunderstood. The critical point is the temperature and pressure above which only a single phase can exist. Supercritical simply means above the critical point.

Supercritical is not recognized as a separate state of matter. There are only gases, liquids, solids, and plasmas. The supercritical region is a transitional region, in which a gas can be converted to a liquid, and vice versa, by changing temperature and pressure, without a phase transition.

The choice of the name *supercritical fluid chromatography* was unfortunate because it does not cover all the fluid characteristics of interest. The conditions used are often subcritical, usually meaning the pressure is above the critical pressure but the temperature is below the critical temperature. Generally, you cannot tell the difference. There is no sudden change in retention, density, efficiency, or linear velocity when the definition of the fluid changes.

Obviously an author would rather not use a technically incorrect name and call something supercritical when it is not. However, stressing the fact that the fluid is not supercritical implies there is something fundamentally different about the two conditions, which is not correct. This confusion has resulted in the creation of many different names for what is essentially the same thing.

During an open discussion at a GC conference in 1957, Jim Lovelock suggested using compressed inorganic gases such as SO₂ as the mobile phase to chromatographically separate highly polar substances. He suggested the name *critical state chromatography*, but never performed experiments. He had a letter notarized in 1958 that still survives, which described what he meant.

Ernst Klesper was the first to actually use a fluid at high pressures and above its critical temperature as a chromatographic mobile phase³⁰. He used fluorocarbon mobile phases to separate porphorins with a packed column. In the first ever SFC paper, he described it as *high-pressure gas chromatography above critical temperatures*. There are a number of papers where the temperature was above the critical temperature, but the pressure, even though high, was below the critical pressure. Fluids under such conditions are by definition a gas, and the technique could be called (high-pressure) *gas chromatography*. However, this name fails to convey the nature of the technique. The fluid acted as a solvent, which is counter

to the general understanding in GC, where the mobile phase is an inert carrier (that is, not a solvent).

In 1966 Giddings published papers on *turbulent gas chromatography* and *ultrahigh pressure gas chromatography*^{31,32}. In another paper, several years later, he changed the name to *dense gas chromatography* to try to differentiate the technique from regular GC, since the mobile phase was acting as a solvent, and the solvent strength was proportional to density³³. Interestingly, the last two papers both used pressures up to 2000 atmospheres. Substantially higher than what currently is considered *ultra*-performance in HPLC.

In 1967 Sie and Rijnders³⁴ were the first to call the technique *supercritical fluid* chromatography³⁴. This is better than calling the fluid a gas, but implies the fluid needs to always be supercritical in order to display the characteristics of interest, which is incorrect.

In 1985 Caude appears to have coined the name *subcritical* fluid chromatography³⁵. By definition, operating at higher than the critical pressure but lower than the critical temperature, the fluid is technically a liquid. However, they rightly concluded that calling it a *liquid* would also be confusing, since the fluid remained highly compressible and needed special hardware such as pumps, and a means to significantly elevate and control the outlet pressure. Nevertheless, the term subcritical continues to be widely used and many people appear to think there is truly a significant difference between sub- and supercritical fluid characteristics.

Olesik coined the name *enhanced fluidity chromatography*, starting in 1991 for a unique variant³⁶. She has also called it *high performance liquid chromatography with enhanced fluidity*. She used CO₂ as a significant fraction of the mobile phase, but at typically 10 to 25 %. She operated at high pressures but below the critical temperature. The fluids retain some of the higher diffusion coefficients, lower viscosity inherent in SFC, but retain more of the polarity inherent in aqueous based fluids. Enhanced fluidity chromatography is a continuum to what others call *subcritical* fluid chromatography. Both groups recognize that by definition, if the fluid is not supercritical it must be a liquid, or *subcritical*.

As is evident from the above, the name of the technique has evolved over time in efforts to try to clarify what the essence of the processes involved is. However, at some point these new names created completely artificial borders or barriers that have no real chemical or chromatographic significance. Calling the fluid a liquid, or a gas, does not capture the full essence of the technique. Calling the fluid supercritical also fails to

describe the processes because, by definition, the fluids with essentially the same properties are subcritical some of the time.

There are several unifying aspects all these names are trying to describe. First, the fluid acts as a solvent, and the solvent strength can be adjusted by changing the composition, or density (temperature and pressure), or both. Second, the fluids remain highly compressible, requiring special pumps, and a means of maintaining high outlet pressures. These two aspects are true of all the fluids of interest, regardless of definition of the fluid at any particular temperature or pressure.

I would like to suggest that all these names describe the same technique, and use the same hardware. A better name might have been *compressible solvent chromatography* but it is much too late now. We have too many names already. Simply allowing *SFC* to describe all these techniques would be a helpful development.

2.1 Why use CO₂?

Today, almost all applications in SFC use CO₂ modified with an organic solvent and sometimes a highly polar additive. CO₂ is the preferred fluid because it is:

- readily available,
- inexpensive,
- has an accessible critical point,
- relatively safe,
- considered *green* since it has been recycled, and
- miscible with a wide range of highly polar modifiers.

2.1.1 Availability

Many fluids have been used as the primary fluid in SFC³⁷. These fluids include nitrous oxide (N₂O), halocarbons, ammonia (NH₃), sulfur dioxide (SO₂), and supercritical water. Some continue to be used to this day. Some are dangerous (nitrous oxide, ammonia). Nothing else comes close to the overall performance of CO₂ although a few fluids have specialty niches.

Massive amounts of CO₂ are used in the beverage industry worldwide. The distribution infrastructure is in place even in relatively remote places. The raw material is a waste product from other industries. These circumstances combined ensure a ready supply at a remarkably low cost.

2.1.2 Cost

Organizations with many SFC instruments, or who perform significant semipreparative SFC, usually install a bulk tank. Such tanks can hold many tons of CO₂ and are usually filled from a large tank truck. At this scale CO₂ costs about US\$ 0.10 per liter. In Dewar flasks and steel cylinders, costs are in the order of US\$ 1.00 per liter. Almost all the increased cost is due to extra handling. Nevertheless, the cost is remarkably lower than most common solvents.

2.1.3 Purity

SFC-grade CO₂ was developed in the late 1980s through early 1990s to provide pure mobile phase for the nascent SFC business. Its introduction was needed at that time, since ordering a cylinder of commercial industrial-grade CO₂ could result in the delivery of a cylinder partly filled with corn oil (author's own experience!). Further, steel CO₂ cylinders used

in the beverage industry were sometimes (inadvertently) backfilled with water or with stale beer, which oxidized the steel, producing colloidal iron particles that were detrimental to the performance, and difficult to clean out. At that time, dip tubes were used to take the liquid CO₂ from the bottom of the cylinder to minimize the chilling power needed. This meant that anything dissolved in the CO₂ also wound up in the chromatograph. These negative experiences in the late 1980s resulted in the development of an ASTM-certified, guaranteed-purity SFC grade CO₂. It is still available. It is typically shipped in 30-pound aluminum cylinders and costs around US\$ 20 per liter, which negates the operational cost advantage of SFC. The primary difference from other grades is that a purity test is run on each batch and a certificate of analysis issued showing it meets certain criteria. There appears to be no reason to buy this grade presently, except perhaps for ultratrace work.

Bulk CO₂ is typically of high purity. Most contamination occurs when the fluid is repackaged in smaller containers. The supply situation has improved dramatically and high-purity industrial grades are usually satisfactory. Many grades are more than 99.99 % pure CO₂. Beverage or food grade is a convenient, safe choice.

It is useful to point out that the Agilent analytical SFC system has a powerful chiller that uniquely liquefies the vapor phase from the cylinder. Thus, an eductor or dip tube, extending from the valve to just above the bottom of the cylinder is not required. By using the vapor phase and not the liquid phase, the fluid is distilled just before use, leaving any nonvolatile contaminants behind in the cylinder.

2.1.4 Accessible critical point

The critical point of pure CO₂ is readily accessible at just over 31 °C and 70 bar. This simply means that CO₂ can be compressed to a dense fluid at relatively low temperatures and pressures. A dense solvent at relatively low temperature is unlikely to damage temperature sensitive, labile compounds. The need for only modest pressures to achieve a dense solvent is convenient and does not impose a significant technical or energy penalty.

2.1.5 Safety

2.1.5.1 Toxicity of CO₂

CO₂ is a product of human respiration and as such is not toxic at low concentrations. Nevertheless, at high concentrations it can be lethal. People should not be alarmed about the presence of CO₂ in the laboratory. Most convenience stores, restaurants, and cafeterias have cylinders of high-pressure CO₂ associated with their soft drink machines. Many fire extinguishers also contain high-pressure CO₂ and are widely distributed

throughout factories and office buildings. The concentration of CO₂ in properly designed SFC laboratories is much lower than is typical in occupied conference rooms or theaters. Large quantities of CO₂ are seldom stored directly in the laboratory, so large-scale escapes are unlikely.

CO₂ is denser than air and can tend to accumulate near the floor of poorly ventilated spaces. Sensors and alarms should be mounted near waist level. Oxygen sensors do not provide needed information, since in any potentially dangerous situation the oxygen level is likely to be near normal, even when there is a dangerous level of CO₂.

2.1.5.2

Fire and explosion hazard

In normal phase HPLC, the mobile phase often consists of heptane mixed with another organic modifier such as isopropanol. Heptane, in particular, is highly flammable and the vapors can be explosive. In many jurisdictions, the amount of such solvents in a laboratory is restricted. In SFC, using CO₂ as the primary component in the mobile phase, it is almost impossible to get the mobile phase to burn.

2.2

Using 100 % CO₂

The solvent strength of pure (100 %) CO₂ is a function of its density. Much of the work in SFC between about 1984 and 1995 employed capillary columns, a flame ionization detector (FID), with isocratic or pressure or density programming with 100 % CO₂ as the mobile phase³⁸. Several applications were also developed using packed columns³⁷. These applications were done with pressure or density programming of pure (100 %) CO₂ and with flame ionization detection, particularly for separation of relatively low-polarity homologous series such as silicone oils and surfactants. Pressure or density programming has largely faded away.

The density of CO₂ changes over a wide range with changes in temperature and pressure as shown in Figure 2.1. At 40 °C, most of the change in density occurs over only a narrow range of pressure between about 70 and 110 bar. Operation in this region means that small changes in pressure produce large changes in density and retention. As the temperature is increased, the curves tend to flatten out, creating a shallower gradient of density against pressure. Most pressure programming has been done at elevated temperatures since, as is obvious from Figure 2.1, it is easier to make small changes in density on a shallower slope.

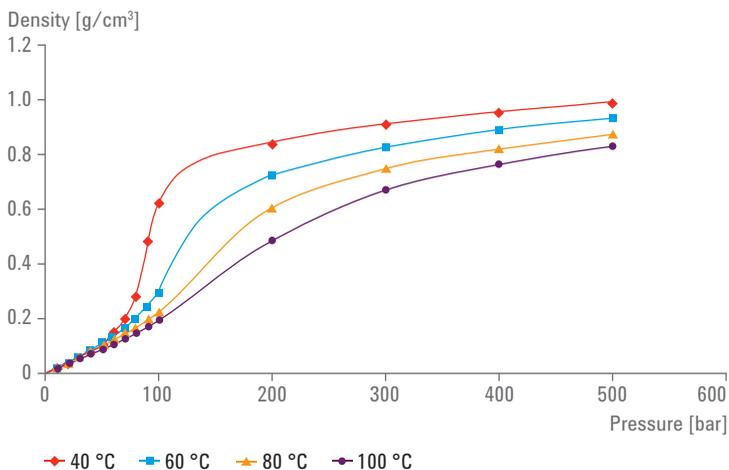


Figure 2.1 Density of pure CO₂ as a function of pressure at four different temperatures. From top to bottom: 40, 60, 80 and 100 °C.

In the petroleum industry, pure CO₂ is still used with packed columns. At low temperatures, such as 28 to 40 °C, with an appropriate silica column, petroleum group separations are performed to determine the aromatic content in diesel fuels for environmental reasons. The method can also be used to determine the aromatics content in crude oil. Under the appropriate conditions, all the aliphatics will elute as a single narrow peak. Olefins also tend to elute as a single peak but often form a tail on the aliphatics. Aromatics elute as a series of peaks, each one representing an increase in ring number. This application has been an ASTM method (D5186) for aromatics in diesel fuel since the mid-1990s and is one of a few techniques that can accurately quantify low levels of aromatics.

A related method (ASTM 7347) uses both the silica column and a silver loaded column with valve switching to better separate the olefins from the aliphatics. The aliphatics and olefins are partially separated on the silica column and allowed to enter the silver column. The aliphatics pass through the silver column, to the FID. The olefins are highly retained and must be back-flushed off the silver column. The aromatics are never allowed to enter the silver column and are directly quantified with the FID. This has become an important method for the determination of olefins in gasoline. Most present applications are not amenable to using pure CO₂ as the mobile phase.

2.3 Modifiers or cosolvents

Today, most SFC applications are performed on relatively polar stationary phases with CO₂ modified with an organic solvent and sometimes other highly polar components, such as acids and bases, called *additives*.

2.3.1 Methanol

Methanol is by far the most widely used modifier and among the most polar modifiers completely miscible with CO₂. Advantages of methanol include:

- availability,
- inexpensiveness,
- complete miscibility with CO₂,
- low UV cut-off (about 205 nm),
- relatively low toxicity.

2.3.1.1 Density of methanol/CO₂ mixtures

The density of pure CO₂ was the primary means of changing retention of low polarity solutes in the past, so changes in density of mixtures should also have some effect on retention. The densities of a few methanol/CO₂ mixtures have been measured^{39,40}. The measured densities were found to increase both with increasing methanol concentration, and with increasing pressure, as shown in Figure 2.2. These values can be used to deconvolute the effects of density and composition on retention by adjusting pressure to maintain constant density, while composition is changed. Such work has shown that there is a small density effect but once a modifier is added, the modifier concentration produces most of the retention change. However, the less polar the modifier, the more pronounced the dependence of retention on density becomes.

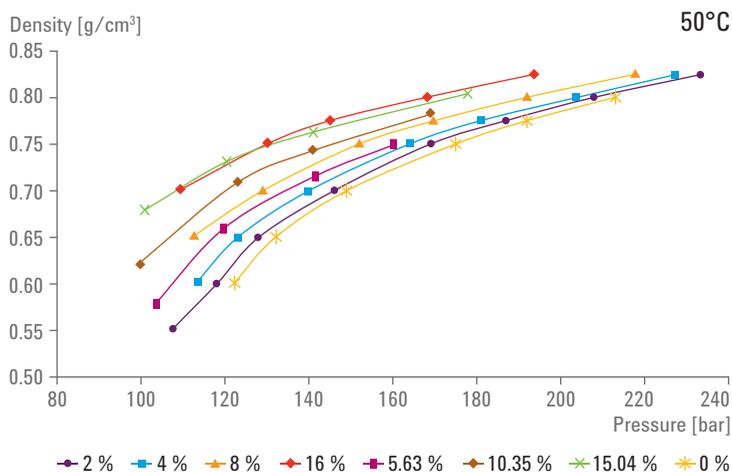


Figure 2.2 Density of MeOH/CO₂ mixtures as a function of pressure at 50 °C. The density of pure methanol is 0.791 g/cm³. The open symbols are from reference 39. The solid symbols are from reference 40.

When changing a method from 5- μ m to sub-2- μ m particles, the pressure drop across the column increases substantially. Thus, the average density in the column is changed. Both retention and selectivity can change noticeably. However, this is usually a secondary effect, as shown in a later section.

2.3.2 Modifier solvent strength scales

Almost any organic solvent can be used as a modifier. Their elution strength roughly follows Snyder's P' elution strength scale^{41,42} or the Hildebrand solvent strength scale for silica⁴³, each developed many years ago for elution from silica in normal phase HPLC. Values for several common organic modifiers are listed in Table 1.1. The actual values have been changed over time. For example, today, methanol is much closer to water than originally thought. The modern P' values for methanol, acetonitrile, ethanol, and isopropanol are 6.6, 6.2, 5.2, and 4.3 respectively. If the same concentration of modifier is used, relative retention tends to follow the solvent strength scale in Table 2.1, but there are exceptions.

Modifier	P'	Hildebrand elution strength (on silica)
Water	9	
Methanol	6.6	0.73
Acetonitrile	6.2	0.5
Methoxyethanol	5.7	
Acetone	5.4	0.47 to 0.53
Ethanol	5.2	0.68
Isopropanol	4.3	0.62
Ethyl acetate	4.3	0.38 to 0.48
Tetrahydrofuran	4.2	0.53
Dichloromethane	3.4	0.32
Methylene chloride	3.4	0.32
Carbon tetrachloride	1.7	
Heptane, CO ₂	0	0.01
Acetic acid	6.2	
Octanol	3.4	

Table 2.1 Snyder's P' values and Hildebrand values for some of the modifiers completely miscible with CO₂.

With a chiral separation⁴⁴, the retention time of the second eluting peak increased from 7.98, to 8.32, to 10.48 minutes when the modifier was changed from methanol, to ethanol, to isopropanol, as expected and as shown in Figure 2.3. The resolution actually decreased with increasing retention times. The same pair of enantiomers was separated using acetonitrile, as modifier, at the same concentration. The retention of the last peak increased substantially to 16.13 minutes, but the separation noticeably improved. It is often the case because acetonitrile is a much weaker solvent than most of the alcohols. On the Hildebrand scale all three alcohols have higher values than acetonitrile, which is consistent with the results in Figure 2.3. Apparently hydrogen bonding is inordinately important in SFC.

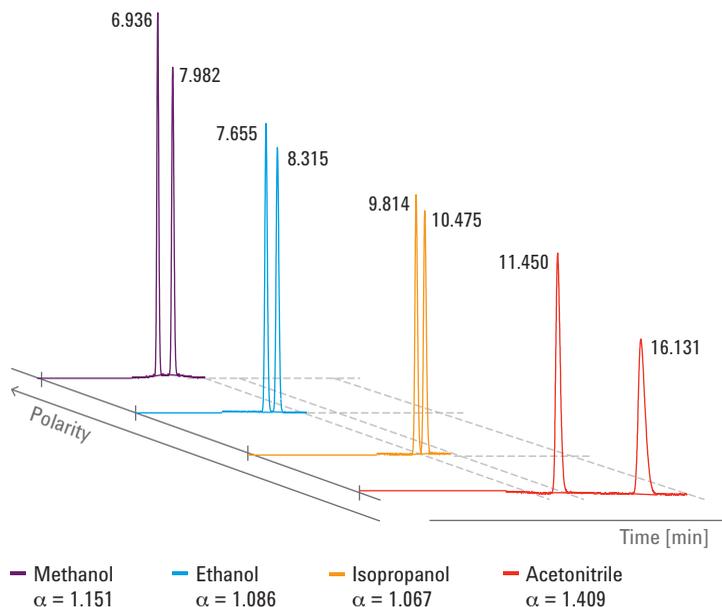


Figure 2.3 A chiral separation showing the increase in retention time as a function of decreasing modifier polarity according to both the P' and Hildebrand solvent strength scales. Acetonitrile is much less polar than expected by P' but not by Hildebrand. All conditions except the identity of the modifier were held constant.

2.3.3 Nonlinear solvent strength

Snyder's P' scale^{41,42} assumes that mobile phase solvent strength (as measured with P') is a linear function of the mole fractions of the two pure liquids, see Equation 2.1 where x is the mole fraction of A .

$$P'_{AB} = xP'_A + (1 - x)P'_B$$

Equation 2.1 Snyder's calculation of mobile phase solvent strength. Does NOT apply to SFC.

Thus, progressively varying the composition of the mobile phase from one pure solvent to the other should produce a linear relationship in solvent strength. However, retention (elution strength) is a nonlinear function of %B. In normal phase HPLC this nonlinearity is explained as due to competitive adsorption between the modifier (B) and the solutes.

In SFC, plots of $\log k$ against %B are also nonlinear, as shown in Chapter 4 "Effect of Mobile Phase Variables on Retention and Selectivity". However, the explanation used in SFC is different.

2.3.3.1 Solvatochromic dyes

A way to measure actual mobile phase solvent strength (as opposed to elution strength) involves the use of solvatochromic dyes. The UV maxima in the absorbance spectra of such dyes changes, depending on the polarity of the solvent the dye is dissolved in. Nile Red is such a dye. The Nile Red energy scale (E_{NR}), calculated from the wavelength of the absorbance maximum, can be plotted against P' , correlating E_{NR} to P' .

Using Nile Red as the solvatochromic dye produced the data presented in Figure 2.4, for pure solvents⁴⁵. The relative order of E_{NR} solvent strengths is consistent with the P' values. Nile Red can also be used to measure the polarity of mixtures of these same modifiers with CO_2 ^{45,46}. A few such results are presented in Figure 2.5. Unlike Snyder's assumed linear relationship, the measured solvent strength is a nonlinear function of modifier concentration. Since there is no stationary phase, a competitive adsorption mechanism *cannot* explain the nonlinearity. Further, substituting the E_{NR} energy scale for %B produces linear plots of $\log k$, at least above about 1 % modifier⁴⁶.

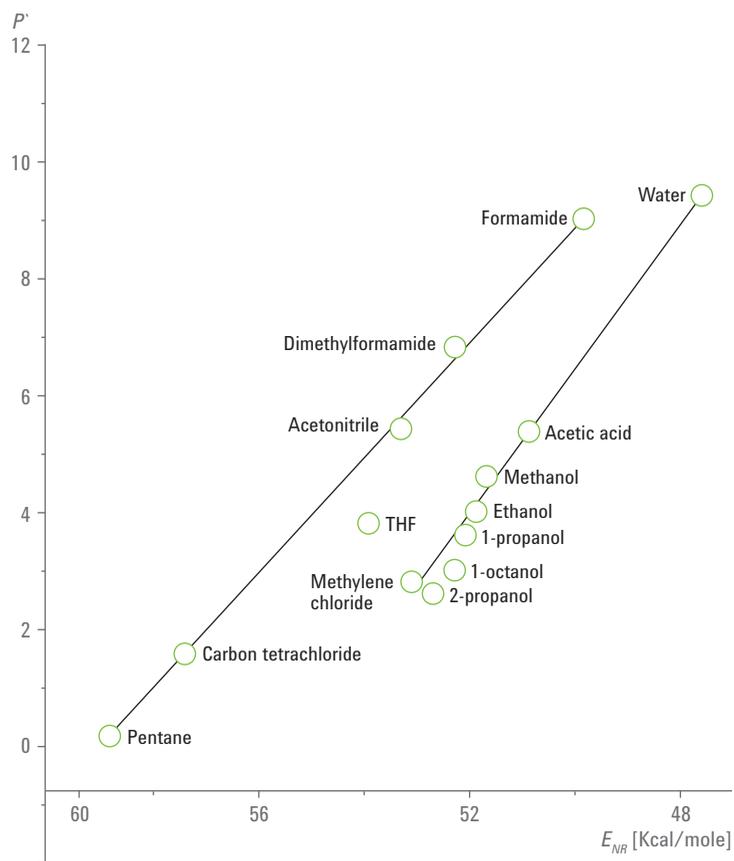


Figure 2.4 Relationship between Snyder's P' scale to the Nile Red solvent strength scale for pure solvents. Note that ACN appears to be more polar than MeOH on the P' scale but much less polar on the Nile Red scale, consistent with the results in Figure 2.3.

Nonlinear Solvent Strength as Measured with Nile Red

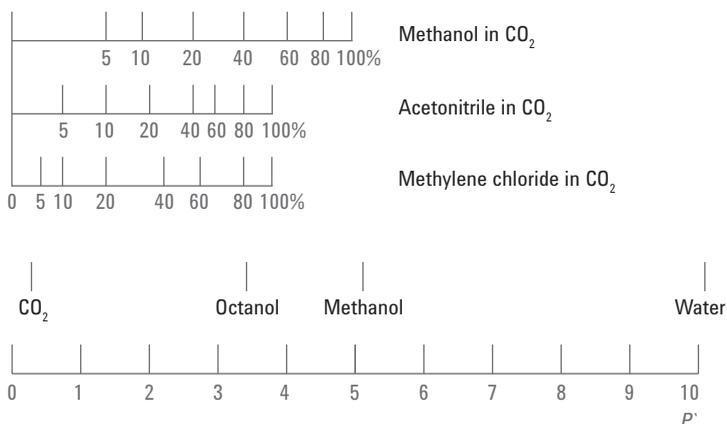


Figure 2.5 Nile Red can also be used to measure the solvent strength of mixtures of CO_2 with mixtures of organic solvents. Again, ACN is shown to be a weaker solvent than MeOH. Note the major nonlinear nature of solvent strength against modifier concentration.

2.3.3.2 Clustering

There is widespread consensus that polar modifier molecules form locally inhomogeneous mixtures with CO_2 . It appears that when the modifier is substantially more polar than the CO_2 , the polar modifier molecules *cluster* together, and in particular, cluster around polar solute molecules⁴⁷. This surrounds the solute particles with a solvation sphere with a higher local modifier concentration than in the bulk of the fluid. These clusters are not as well defined as a micelle.

2.3.3.3 Adsorption on the stationary phase

Using tracer-pulse MS experiments it has been shown that the first small additions of polar modifier to CO_2 results in extensive adsorption of modifier molecules onto polar stationary phase. In fact, about 1 % methanol was shown to produce several monolayers of modifier coverage on a diol column⁴⁸. At higher concentrations only a relatively small additional amount was adsorbed. This coverage by the modifier tends to change the chemical nature of the stationary phase.

At the same time, multiple monolayers of CO_2 can also be adsorbed⁴⁹. Thus, there is a deep *pool* of high-density modified fluid adsorbed on polar stationary phases, the composition of which depends on temperature, pressure and composition⁵⁰. These effects have not been studied in detail.

2.3.3.4 Changing selectivity with the modifiers

The absolute value of the elution strengths listed in Table 2.2 is only part of the solvent strength picture. Snyder also developed a solvent selectivity classification scheme starting with Rohrschneider's gas-liquid partition coefficients for ethanol, dioxane, and nitromethane with 82 solvents^{41,42}. It was assumed that Van der Waal forces were universal. Consequently, he plotted the data on a triangular graph, each side representing the ability of the solvent to interact with proton donors, proton acceptors or a dipole. A simplified version is represented in Figure 2.6. The solvents tended to clump together in groups that Snyder characterized as eight solvent families, the members of each group having similar selectivity. Changing modifier from one selectivity family, keeping the P' of the modifier constant, tends to not change absolute retention significantly but does change selectivity. In other words, if one modifier fails to provide adequate separation of closely related compounds, try a modifier from a different solvent family, instead of another from the same family.

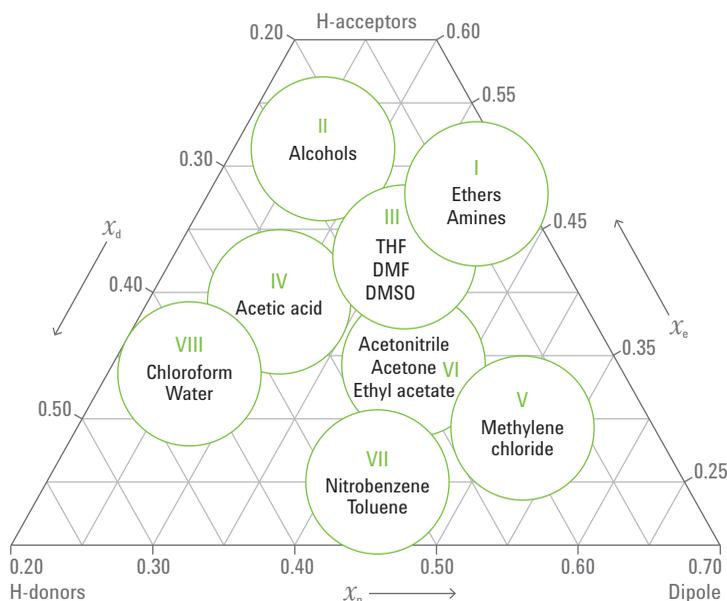


Figure 2.6 Snyder's solvent triangle, breaking down solvents into eight families. To change selectivity significantly, switch to a modifier in a different solvent family.

Additive	Comment
Isopropylamine	Reacts with some ketones
Dimethylethylamine	Volatile but stinks
Triethylamine	Sometimes less effective due to steric hindrance
Many other amines	
Ammonia	Relatively recent
Tert-alkylammonium salts	
Alkylsulfonic acids	
Trifluoroacetic acid	
Formic acid	
Acetic acid	
Citric acid	
Ammonium acetate	
Ammonium formate	
Water	

Table 2.2 Additives widely used in SFC.

2.4 Additives

Many strong acids and bases, and most amphoteric compounds do not elute or elute with poor peak shapes when eluted with binary mixtures of CO₂ and a modifier such as methanol or ethanol. The addition of a small amount of a highly polar additive^{51, 52}, dissolved in the modifier usually dramatically improves peak shapes, as shown in Figure 2.7. The concentration of additives is usually between 0.1 and 2 % in the modifier.

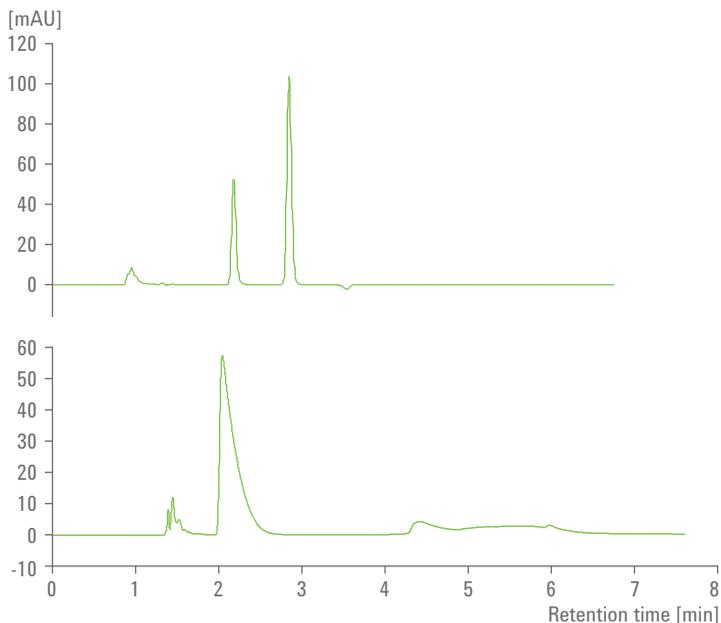


Figure 2.7 The effect of additive on peak shape and elution using the drug Adderall, as an example. Adderall is a nonracemic mixture of the enantiomers of amphetamine with excess of the D enantiomer. The lower chromatogram represents elution without additive; the upper chromatogram with triethylamine. All other conditions unchanged.

Many times a strong acid improves the peak shapes of strong acids, while strong bases usually improve the peak shapes of other strong bases. However, this is only a generalization and the opposite is sometimes true⁵³ (acid for a base and vice versa), probably as a result of something like ion pairing in HPLC. Amphoteric solutes are often the most difficult and may require multiple ions or counter ions. A list of common additives is included in Table 2.2. Additives can:

- suppress solute ionization,
- cover active sites on the stationary phase, and
- form neutral ion pairs with the solutes.

Additives strongly adsorb onto polar stationary phases⁵⁴ sometimes forming near mono-layers, even at concentrations well below 1 % in the modifier (much, much less than 1 % in the total mobile phase). Often no additive elutes for some minutes after the additive is first introduced into the mobile phase, particularly at low modifier concentrations. Care should be taken to wait long enough for the additive concentration to equilibrate.

Additives are not always washed off after they are removed from the mobile phase. The use of additives can permanently⁵⁵, or at least temporarily changes column characteristics. It is often true that even after removal of the additive from the mobile phase, peak shapes tend to remain improved for some time, although there may be a slow degradation of performance back toward the performance initially observed without additive. Such apparently irreproducible but predictable behavior can complicate screening, or require the use of several sets of identical columns, one for use with an acidic additive and another for use with a basic additive, for example. Switching back and forth between acidic and basic additives without an intermediate wash should be avoided.

Columns should never be stored without a careful wash after the use of an additive, particularly amines. In the author's lab, after using a basic additive, the column is washed for about 10 column volumes, with 40 % methanol in CO₂, followed by 40 % (methanol + 0.2 % acetic acid) in CO₂, followed by a rinse with 40 % methanol in CO₂. The acetic acid, being a relatively weak acid, can displace or neutralize strongly adsorbed basic additives from the surface of the stationary phase, yet can usually itself be washed off with CO₂/methanol leaving a relatively bare stationary phase without adsorbed polar additives.

Other additives sometimes desorb with pure organic solvents. In extreme cases, isopropanol has been used followed by an isopropanol -water mixture, followed by isopropanol, in order to remove polar additives.

Columns should, at the very least, be rinsed with 40 % modifier in CO₂ for greater than 3 to 10 column volumes before storage. After washing, the columns can be stored wet or dry. In the author's lab, the columns are vented to atmospheric pressure, meaning the columns are not filled with a liquid solvent. Then, the columns are stored without plugs inserted in the column end fittings, after the column is removed from the system.

In recent years, there have been concerted attempts to develop stationary phases that do not require additives in the mobile phase. These phases will be discussed in more detail in Chapter 3 "The Stationary Phase".

Additives can interfere with mass spectrometric (MS) detection, particularly if they have poor volatility. When using a mass spectrometer for detection, it is important to choose an additive that is quite volatile, such as ammonium acetate or ammonium formate, or even ammonium hydroxide.

2.5 Extending solute polarity with added water

Normal phase HPLC is not ideal for use with aqueous samples, particularly when the mobile phase is nonpolar. Small amounts of water, from traces in the modifier or from an aqueous sample, tend to preferentially adsorb onto the polar stationary phase. Re-equilibration tends to be slow. Some older reports suggest it takes up to 1000 column volumes to re-equilibrate. Consequently, aqueous samples are usually avoided. SFC has none of these issues, since the low polarity CO_2 is miscible with much more polar solvents, compared to hexane or heptane. These more polar modifiers can help solvate small amounts of water.

Water is only soluble to a few tenths of 1 % in pure CO_2 . However, when methanol is mixed with CO_2 , at least 10 % water can be added to the methanol without significantly distorting peaks of very polar compounds. Additives can still be added. Nucleic acids and nucleosides were rapidly separated⁵⁶ on an amino column using 10 % water in methanol with ammonium acetate and formic acid added. Equilibration time remains fast. The addition of water enables the separation of solutes that are more polar than possible using just methanol. Further, aqueous samples, can be diluted with methanol 3:1 or 4:1 and injected directly, without loss of retention time or area reproducibility.

3.1 Materials

The vast majority of SFC applications have used totally porous, very high purity, bare silica or bonded phases on the same totally porous silica. There are no issues with particle strength, since the particles are not subjected to ultrahigh pressures or high pressure drops. There are only a few reports on the use of other materials such as porous shell, monolithic columns or zirconia based particles.

Today totally porous particles are almost always spherical with diameters between 1.7 and 5 μm . Larger particles are in decreasing use, even for semipreparative scale, with up to 5-cm id columns. The particles that give the best retention and peak shape, at least for polar solutes, are usually totally porous, smaller pore, higher surface area particles with pore sizes between 60 and 120 Angstroms, and surface areas greater than 350 m^2/g .

3.2 Achiral bonded phases

3.2.1 Nonpolar phases

Much of the early work in SFC involved relatively non-polar solutes, which were best separated on a nonpolar column such as C18. A few workers characterize this work as *reversed phase* SFC, specifically when the separation involved a C18 or similar stationary phase and moderately polar modifiers. However, the mobile phase usually consisted mostly of CO_2 and a highly nonpolar solvent similar to heptane in solvent strength, which were mixed with a more polar modifier. The modifier concentration was almost always programmed with increasing modifier concentration. Thus, the mobile phase was being programmed from lower to higher polarity and hence definitely not reversed phase.

Reversed phase HPLC, clearly one of the most dominant chromatographic techniques with hundreds of thousands of practitioners, is most often used with C18. Consequently, there are many such high-quality columns available. These columns can also be used for SFC. The C18 phases work well with triglycerides, surfactants, silicone oils, carotenoids, other terpenoids, and many compounds with a long aliphatic tail. With polar solutes there is generally little retention and often poor peak shapes.

3.2.2 Polar phases

SFC is generally performed as a normal phase technique, where polar stationary phases are used and the mobile phase is programmed from low to higher polarity. Although HPLC started out as a normal phase technique, it was long ago supplanted by reversed phase in just about every application area except for chiral separations and older Pharmacopeia methods. Subsequently, until recently there has been little effort by column manufacturers toward making newer polar stationary phases.

Traditional polar normal phase stationary phases include several forms of bare silica, cyano, amino, and *classic* diol. Chromatograms of a mix of caffeine, theophylline and theobromine demonstrate the differences in selectivity between C18, cyano, bare silica, amino and diol, as shown in Figure 3.1. These moderately polar compounds were virtually unretained on the nonpolar C18 even at a low (8 %) methanol concentration. In contrast, they were well retained and separated on a cyano column under the same conditions. The bare silica column was more retentive, requiring higher modifier concentrations (18 %) to achieve similar retention times. Selectivity was similar to cyano. The amino column actually created a reversal in elution order (1, 3, 2 instead of 1, 2, 3) but the resolution between 2 and 3 was still not ideal. The diol phase was a bit more retentive than the amino phase, without the peak reversal, but the peaks were slightly better resolved due to differences in selectivity. These chromatograms indicate a significant variation in relative retention based solely on the identity of the bonded phase. The chromatograms presented were collected some years ago, using the same high-purity, silica-based material. Nevertheless, the results are representative of modern results, although modern diol columns are more like bare silica.

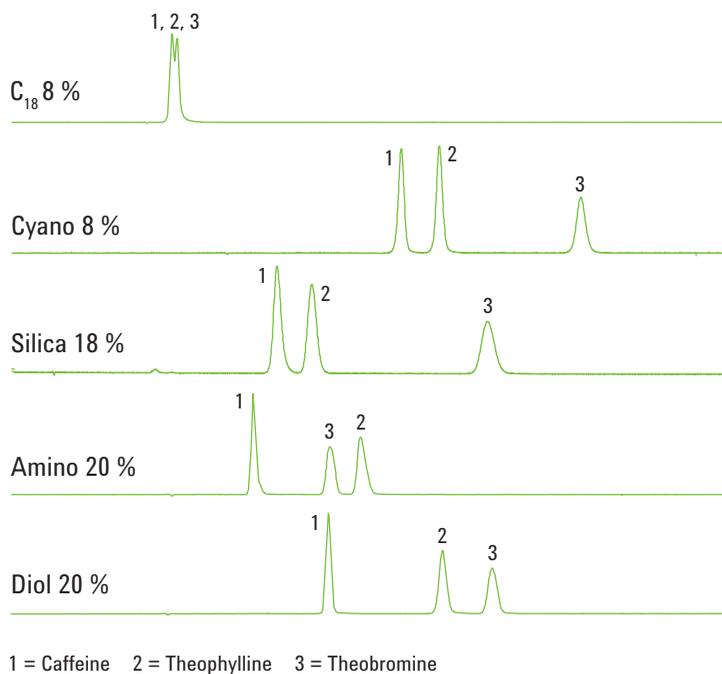


Figure 3.1 Retention and selectivity of caffeine (1), theophylline (2) and theobromine (3) on various stationary phases.

Both SFC and hydrophilic interaction liquid chromatography (HILIC) have renewed the interest of column manufacturers for creating new polar phases. The HILIC columns appear to be similar to bare silica but are sometimes called diol.

Most of the innovation has come from smaller column manufacturers trying to meet the niche needs of SFC users. As indicated in Chapter 2 “The Mobile Phase”, it is often necessary to add an additive to the mobile phase, particularly with aliphatic amine solutes. Several ethylpyridine phases have been developed specifically for separating amines, which often minimize, or eliminate, the need for an additive. Other proprietary phases have also been developed for separating acids without an additive. These newer phases tend to be more retentive than the classic polar phases, requiring a higher modifier concentration, which makes them slightly less desirable. A partial list of the more common phases is presented in Table 3.1.

Agilent	Other suppliers
RX-SIL (5, 3.5, 1.8 μm)	Ethylpyridine (5, 3, 1.7 μm)
SB-CN (5, 3.5, 1.8 μm)	Premier (5, 3 μm)
Hexylphenyl (5, 3.5, 1.8 μm)	Diol (5, 3, 1.7 μm)
HILIC (5, 3.5, 1.8 μm)	Many others
SB-C18 (5, 3.5, 1.8 μm)	
Eclipse C18 (5, 3.5, 1.8 μm)	
XDB C18 (5, 3.5, 1.8 μm)	
Amino (5 μm)	

Table 3.1 Common stationary phases.

It is worth mentioning that it is relatively difficult to get more than one version of some of these polar phases with smaller particle sizes. These include diol and amino. However, HILIC, bare silica, cyano, hexaphenyl and C18 are all readily available in 1.8 and other sub-2- μm particle sizes.

3.2.3

Too many phases?

In SFC, we have a wide range of good stationary phases with variable selectivity to try to resolve difficult peak pairs, although it usually *is not necessary*. It is fairly simple to match a stationary phase to the solutes, as suggested by Figure 3.2. There are usually a number of different stationary phases that could be used for any specific separation, any one of which could be chosen, and which will probably yield a viable separation.

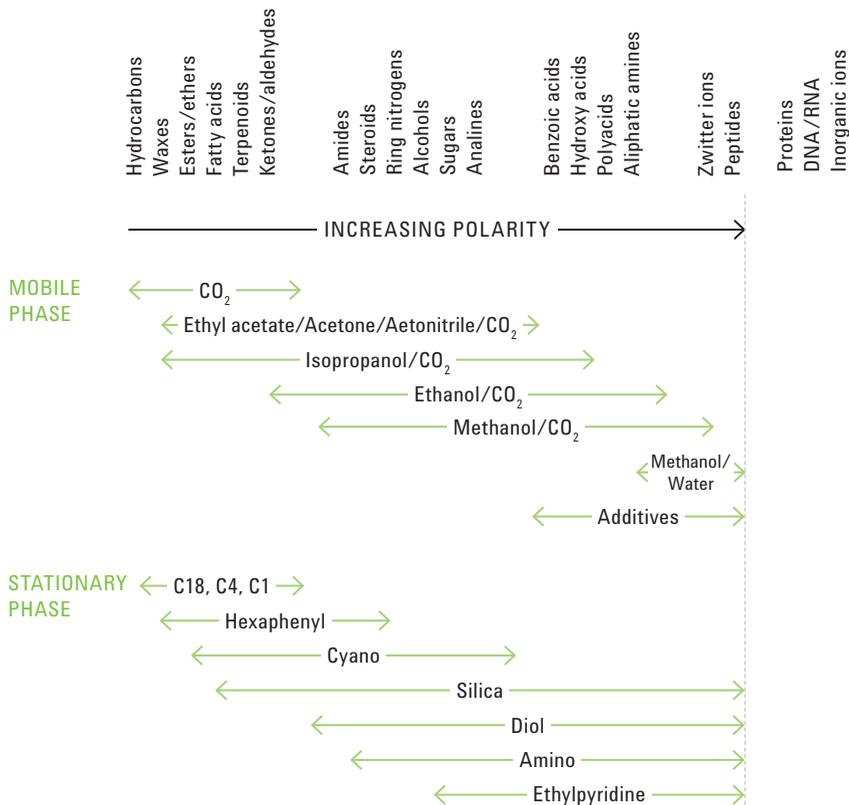


Figure 3.2 Specific mobile and stationary phases are matched with solute families. Progressively more polar solutes require progressively more polar mobile phases and stationary phases.

For polar solutes, just about any of the polar phases will do. A method can generally be optimized by changing mobile phase composition, temperature and pressure as outlined in Chapter 4 “Effect of Mobile Phase Variables on Retention and Selectivity”. The only time you need to change the stationary phase is in situations when changing the mobile phase parameters fails to produce adequate selectivity.

3.3 Stationary phase comparisons

Lesellier⁵⁷ developed a method for visualizing differences in column selectivity based on a solvation parameter model, vaguely akin to Snyder's solvent triangle, except it applies to stationary phases, not the mobile phase. In this case, five parameters were used to generate a spidergram, in which columns were evaluated and compared. A simplified version is presented in Figure 3.3. As can be seen, all the classic polar phases such bare silica, cyano, amino, and diol cluster fairly closely together across the axis of proton donors. Diol has a tendency to also be a proton acceptor more than the others, while cyano and amino tend toward dipole-polarizability characteristics. The relatively new pentafluorophenyl (PFP) has not been widely used in SFC. In this model, it represents dipole-polarizability, something akin to some of the chlorinated solvents in Snyder's triangle.

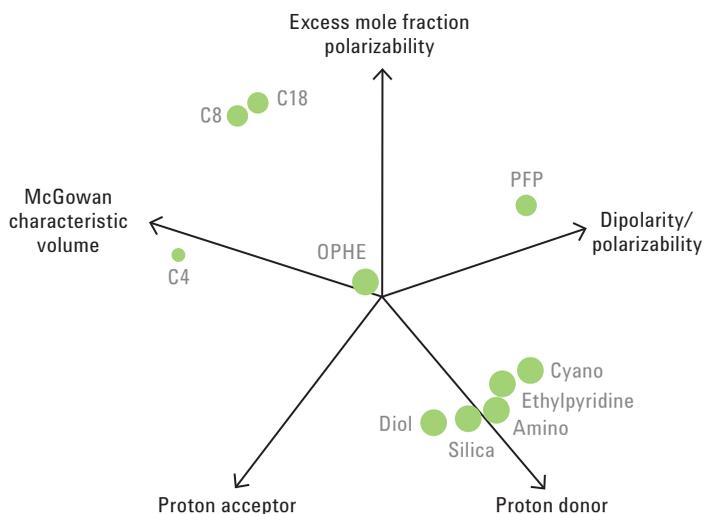


Figure 3.3 Column selectivity using Lesellier's solvation parameter model. OPHE: SynergiPolar RP (phenyl-propyl) Phenomenix, EP: 2-ethyl-ethylpyridine, PFP: pentafluorophenyl-propyl (Supelco).

The hydrocarbons C8 and C18 are clustered together, more or less directly opposite to the classic polar phases, as one might expect (reversed phase against normal phase). The relatively new phenyl-propyl phase appears to be relatively neutral, placing it near the center of the plot directly between the nonpolar aliphatic hydrocarbons (C8, C18) and the polar classic columns (bare silica, cyano, amino or diol). C4 is removed from the other aliphatic hydrocarbons. Again, if one stationary phase provides poor separation, it is probably appropriate to switch to a different stationary phase group.

3.4 Relationship between particle diameter and column dimensions

Older SFC applications were largely performed using 4.6 by 250-mm columns packed with 5- μm totally porous particles. Instruments capable of producing the full efficiency of columns packed with 3 or 3.5- μm particles have been available for several decades, but such particles were not often used in SFC. Even 10 years ago, a large part of SFC achiral applications involved rapid screening, with 4.6 by 50 to 150-mm columns, but still mostly with 5- μm particles.

Sub-2- μm particles have been around for over a decade but mostly in the form of C18 for use in UHPLC. Such particles offer a three to four-fold improvement in speed, compared to 3- μm particles, or about a nine-fold advantage over 5- μm columns, at the same efficiency, when each is operated at optimum velocity. Decreasing the particle diameter decreases the run time in two ways. The optimum flow rate and the efficiency are both inversely proportional to d_p . Decreasing the particle size by a factor of two requires twice the flow rate, and generates twice the efficiency in the same length. Cutting the length in half generates the same efficiency as the column with larger particles but in one-quarter of the run time. Since the C term in the van Deemter Equation is rather flat, much of the early UHPLC literature stressed using even shorter (30 to 50-mm) columns operated substantially above optimum velocity to increase speed even further.

There is no reason why such particles could not also be used in SFC, provided appropriate phases are available, and the instrumentation has appropriately low dispersion. Although used for a decade in UHPLC, sub-2- μm particles have only started to be used in SFC, over the last few years⁵⁸. Initially only bare silica was available. However, in recent years several of the classic polar phases, and 2-ethylpyridine have become available in this format. Both the column id and the particle diameter (d_p) have been decreasing rapidly. Columns with 3-mm id and 100-mm length, packed with sub-2- μm particles are becoming a de facto standard.

3.4.1

Pressure drops, thermal gradients and loss of efficiency

The pressure drop across a column is a function of d_p^2 , at constant efficiency. Thus, the pressure drop using 1.8- μm particles is about 7.7-times higher than the pressure drop using 5- μm particles, with equal efficiencies, at the optimum velocity for each. Operating at double the optimum velocity further increases ΔP to greater than 15 times. The resulting high pressures required the redesign of many pumping systems to perform UHPLC.

3.4.1.1

Thermal gradients in UHPLC

High pressure-drops were predicted to cause axial and radial temperature gradients from the earliest days of HPLC⁵⁹⁻⁶¹, and were found to be a problem⁶² when using sub-2- μm packing under extreme conditions. The mobile phase heats up as it expands, and temperature at the column outlet can be significantly above the column compartment set temperature. The gradient along the axis is not considered a problem. However, if there is significant heat transfer from the mobile phase into the column compartment, radial thermal gradients can form along the column. Such radial gradients can result in lower viscosity and higher velocity at the column axis, compared to higher viscosity and lower mobile velocity near the wall. Thus, different parts of the same peak could travel down the column at different speeds, broadening the peak, and causing a loss of efficiency. The existence of such radial gradients is a major justification for the use of 2.1 or even 1-mm id columns, since the shorter distance between the column axis and the wall should decrease the amplitude of the radial temperature of the gradient.

There are major instrumental consequences and considerable debate over this topic. Others have used 4.6-mm columns with sub-2- μm particles at up to 600 bar without significant efficiency losses. This may be due to the way column temperature is controlled. The mobile phase can be heated to the working temperature before entering the column, with the column in a stagnant air bath oven and insulated from the heat exchanger. This minimizes heat transfer out of the column and along the column, since both the stainless steel hardware and stagnant air are poor heat conductors. Under such conditions, the column acts largely adiabatically. This enables the unimportant axial gradients to form but minimizes any problematic radial gradients.

3.4.1.2

Thermal gradients in SFC

Similar arguments about efficiency losses due to thermal gradients have also been made in SFC^{63,65}. If the column is placed in a water bath, the full length of the stainless steel hardware can be maintained at the same temperature. The axial gradient will form, but now the difference in temperature between the axis, caused by expansion, and wall will result in a radial gradient, and serious losses in efficiency have been demonstrated. Therefore, just as in UHPLC, it is often recommended that 2.1 or even 1-mm id columns be used.

However, most of the relevant experiments were done using pure CO₂, or 5 % methanol in CO₂. Under those conditions, the mobile phase cools down significantly on expansion. Unlike UHPLC, temperature gradients in SFC, due to Joule-Thomson (J-T) expansion, are strongly dependent on the composition of the mobile phase. For pure CO₂, expansion from 600 to 100 bar would result in a temperature drop of 16 degrees across the length of the column, as noted in those reports. At 5 % methanol, the maximum temperature drop would still be about 10 degrees. However, at 10 % methanol, the fluid first warms 2 to 3 degrees before falling 5 degrees. Above about 18 % the fluid actually heats up slightly, while at 50 % methanol the fluid never cools and heats up by 10 degrees.

Methanol concentration [%]	T_{out} [°C]	ΔT
0	40	-10
5	44	-6
10	47	-3
20	52	+2
50	60	+10
100	65	+15

Table 3.2 Theoretical adiabatic temperature drop across a column, assuming the inlet was at 600 bar and 50 °C, and the outlet was at 150 bar.

Remember that the pressure drops in SFC are one-third to one-fifth as large as in UHPLC. Although some instruments are capable of 600-bar operation, such high pressures are almost never required. More typical operation is with an outlet pressure at 150 bar and with an inlet pressure between 200 and 350 bar. Under these conditions, an axial gradient would be no more than a few degrees. The Agilent thermostatted column compartment is a still-air column compartment, in which a heat exchanger is used to preheat the mobile phase before entering the column and the column can easily be insulated from the heat exchanger. As a result, there is little radial gradient formed and little efficiency loss due to this phenomenon, under practical conditions. This creates little incentive to use columns 2.1-mm or 1-mm id in SFC, as is sometimes recommended in UHPLC and SFC.

Using sub-2- μm particles in 2.1 or even 1-mm id columns puts severe constraints on the design of the chromatograph in terms of extracolumn band broadening. For example, a 2.1-mm column packed with sub-2- μm particles requires a detector cell with a volume of less than 1 μL , along with short (less than 60 cm) connector tubing of 125 or even 100- μm id. Currently there is no commercially available instrument that can produce more than about half the theoretical efficiency of a 2.1 by 100-mm column packed with sub-2- μm particles, *using standard configurations*. Using such columns requires a major redesign or replumbing, or both, of all existing SFC systems. However, it is both feasible and desirable to use larger id columns that decrease extracolumn band broadening requirements, making it possible to see most of the efficiency of sub-2- μm particles with existing hardware with only modest modifications.

In the other direction, there is also little incentive to use 4.6-mm id columns with sub-2- μm particles, since, in SFC, the optimum flow rate on a 4.6-mm column approaches 5 mL/min. This is the maximum flow rate of the Agilent analytical SFC system. Some other SFC systems have a lower maximum flow rate, particularly at higher column head pressures, and would need to operate suboptimally much of the time. Thus, column inside diameters significantly smaller than 4.6 mm are desirable, and are necessary for operating above the optimum velocity or flow rate with sub-2- μm particles. Since both 2.1 and 4.6-mm id columns have issues with either extracolumn band-broadening or with flow rate limitations, a reasonable compromise seems to be a 3-mm id column size. With a 3 by 100-mm column packed with 1.8- μm particles, the Agilent analytical SFC system can be easily reconfigured to allow about 90 % (or more and at least 96 %) of the theoretical efficiency of the column to be observed, as demonstrated in Section 3.5.2 "High-speed work".

3.5 Recommended column dimensions

3.5.1 Routine work

For new methods, there is no longer any obvious advantage to using totally porous 5- μm particles in SFC for analysis. There is no aspect of speed, resolution or sensitivity that is not exceeded by using smaller particles in shorter, faster columns. Instrumentation and columns are readily available that produce equivalent performance, but much faster, and potentially with higher sensitivity, on smaller diameter particles. For validated methods, 5- μm particles, in 250-mm columns, will continue to be used.

Columns of 4.6 mm in diameter, packed with 3 or 3.5- μm particles are compatible with larger UV detector flow cells, such as those with 10-mm path length and 13- μL cell volume that are widely used in Agilent's multiple-wavelength and diode-array detectors. The long path length of the UV detector cell retains or improves on the sensitivity observed with 5- μm particles, since, with the smaller particles and higher flow, the peaks are narrower in time with greater height.

The tubing connecting the injection valve to the column, and the column to the detector, can collectively be over a meter long with 175- μm id. With such a system, nearly 100 % of the theoretical efficiency of the column can be observed. The 175- μm tubing minimizes the extracolumn pressure drop across the system.

For routine work, a column of 4.6 by 150 mm, packed with 3 or 3.5- μm particles is recommended. Methods developed on such columns should be robust and yield results similar to the best achieved with HPLC. Retention time reproducibility should be much less than 1.0 % RSD ($n=10$) in all but the most extreme cases, and it is often true that RSDs less than or equal to 0.1 % are achieved. Area reproducibility depends on signal-to-noise. For a peak with signal-to-noise greater than about 100, area RSDs should be below 1 %. For much larger peaks, RSDs should be well below 1 %, sometimes approaching 0.1 %. Linearity in calibration curves generally exceeds 0.999, as shown in later sections (see, for example, in Chapter 8 "Quantification in SFC").

3.5.2 High-speed work

For high-speed work, sub-2- μm particles can be used. Agilent's sub-2- μm packing is 1.8 μm . As mentioned previously, 4.6-mm id columns packed with 1.8- μm particles have an optimum flow rate of about 5 mL/min, which is the maximum flow rate of the Agilent analytical SFC system, leaving little or no margin for adjustment. Decreasing the column inside diameter to 3 mm drops the optimum flow rate to about 1.7 to 2 mL/min, leaving plenty of margin for super- (above)-optimum flows. This lower flow rate (compared to the use with 4.6-mm id columns) significantly decreases the pressure drop in the extracolumn components of the system, such as the connector tubing. However, using both the smaller particles and a smaller inside diameter requires significant decreases in extracolumn dispersion. Much of the standard 175- μm id connector tubing in the Agilent analytical SFC system must be replaced with the shortest possible lengths of 125- μm id tubing. Further, the standard 10-mm, 13- μL flow cell must be replaced with a new 3-mm, 2- μL high-pressure flow cell. These rather simple changes enable about 90 % of the theoretical efficiency of the column to be observed, even at k equal to about 2 to 3. A column of 3 by 100 mm packed with 1.8- μm RX-SIL has been shown to produce 120 % of the efficiency of a 4.6 by 150-mm column packed with 3.5- μm RX-SIL at three times the speed, as shown in Figure 3.4.

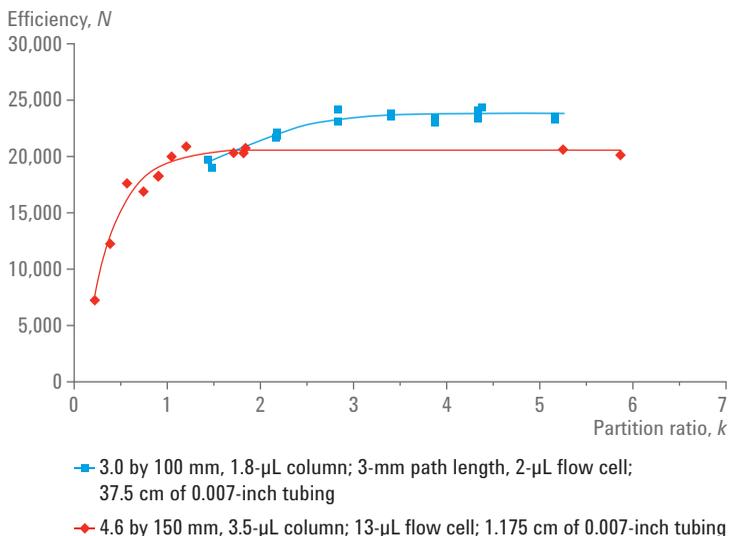


Figure 3.4 Measured column efficiencies. The upper curve was obtained using a 3 by 100 mm, 1.8- μL column, with a 3-mm path length, 2- μL flow cell, 37.5 cm of 0.007-inch tubing (standard heat exchangers) and 80 cm of 0.005-inch connector tubing. The lower curve was obtained with 4.6 by 150 mm, 3.5- μm column with a 13- μL flow cell and 1.175 cm of 0.007-inch tubing. Although the smaller column exhibits poorer peak fidelities, it produced about 20 % higher absolute efficiencies in less than one-third of the time.

3.5.3 Columns of less utility

Porous shell particles have not yet been widely used in SFC, although several reports have appeared^{66,68}. It has been demonstrated that some larger, superficially porous particles, can generate significantly higher efficiencies at higher speeds than totally porous particles of the same diameter⁶⁶. Such larger particles can rival sub-2- μm totally porous particles for efficiency and speed, but with a fraction of the pressure drop. Such columns have been shown to produce sub-2- μm -like performance in HPLC when using 400 to 600-bar chromatographs. This is of great interest in HPLC, but not important for SFC, since the pressure drops with totally porous sub-2- μm particles are already similarly low.

One aspect of porous shell particles that needs further investigation is sample capacity. In one study⁶⁶, using various polar solutes and polar phases, reduced plate heights to about $1.6 d_p$, but only with small injection volumes less than 0.5 μL and on a 4.6-mm id column. The sample solvent was methanol. In another report⁶⁷, using a C18 phase with nonpolar solutes, higher injection volumes (up to 3 μL on 4.6-mm columns) could be used before there was any significant loss of efficiency. The sample solvent was, in this case, methanol/methylene chloride, but the solutes were highly nonpolar. Another paper⁶⁸ by the same authors showed a steady decrease in efficiency with increasing injection volume, again using C18 porous shell columns.

These anomalies appear to be related to the strong solvent effect, where the sample solvent is stronger than the mobile phase. Injecting larger volumes of such a strong solvent is known to distort early eluting peaks, particularly on columns with low surface area.

A relatively small number of SFC papers have been published using these particles with similar results. No useful generalization about injection volume against column inside diameter, modifier concentration, or sample solvent can be made. However, it is fairly simple to recognize the problem and possibly correct it by using a less-polar sample solvent. Any form of solventless injection, where the sample solvent is removed and the solutes are then redissolved in the mobile phase, eliminates this problem.

3.6 Columns for chiral separations

Virtually all the types of chiral stationary phases have been used effectively in SFC. Pirkle-type, cyclodextrin-based and amino acid-based stationary phases have all been used fairly extensively. However, by far the most nearly universal phases have been made with derivatized macrocrystalline cellulose or amylose coated on silica. Patents on several of these highly successful phases have expired and there are now several manufacturers producing them. This has spurred the development of bonded versions that enables the use of a wider choice of modifiers.

The coated phases, because they were macrocrystalline, were usually coated on particles with large pores (1500 Angstroms or more). This yielded low surface areas and relatively poor sample capacity. Some of the other types such as Pirkle-type phases could be bonded to silica with much smaller pores, yielding significantly higher surface area and sample capacity. This is most important in semipreparative work where throughput is the highest priority.

Analytical SFC was largely responsible for the shift from 10- μm particles as standard to the ubiquitous use of 5- μm particles, starting 10 to 15 years ago. In the last few years, 3- μm particles have become common. Column length, and to some extent inside diameter, have both decreased as a result. There are only a few reports^{65,67} of the use of sub-2- μm particles for chiral separations in UHPLC, and none in SFC. This will likely change in the future.

As in achiral analysis, the tendency in SFC is toward rapid screening, sacrificing efficiency for speed. The current tendency is to use 50 to 100-mm columns with 3- μm particles. Columns 150 to 250-mm long, with 3 to 5- μm packing are more often used for trace analysis (enantiomeric excess, EE, determinations). For more in-depth discussions, refer to Chapter 7 "Chiral Separations".

SFC software provides the analyst with four mobile phase control variables:

1. modifier concentration,
2. temperature,
3. pressure, and
4. flow.

Each variable affects retention and selectivity to a different extent.

4.1 Modifier concentration

Among the mobile phase control variables, adjustment of the modifier concentration provides the most powerful control over retention, but less over selectivity.

As a *rule-of-thumb*, doubling modifier concentration halves retention.

Two examples^{69,70} from the literature of the elution of families of relatively polar compounds, hydroxysteroids and tricyclic antidepressants, are shown in panels A and B of Figure 4.1. As predicted from the solvatochromic dye studies in Chapter 2 “The Mobile Phase”, the first small additions of modifier can have a large effect on retention. In the first example, the change in retention of the hydroxysteroids above 5 % methanol is minor, as shown in panel A of Figure 4.1. However, between the first few tenths of 1 to 2 %, the partition ratio, k , changed from nearly 40 to less than 5. The plot is a logarithmic plot of k against percent modifier and is nonlinear. Such large shifts in retention with small changes in modifier concentration make reproducibility, over time and between labs, difficult. Consequently, such conditions are avoided. Nevertheless, such an example shows the power of changing modifier concentration.

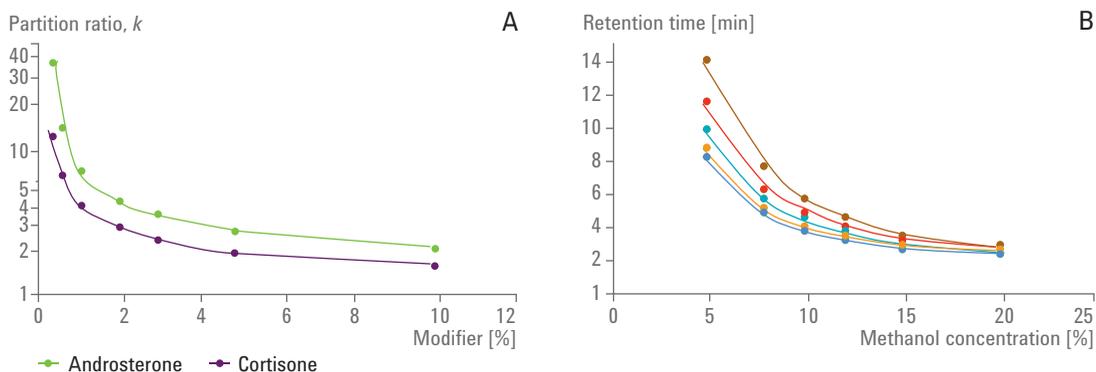


Figure 4.1 A: Change in retention as a function of modifier concentration for cortisone and androsterone, showing large changes with small changes in concentration at low concentrations. B: Change in retention of five tricyclic antidepressants as a function of modifier concentration at higher modifier concentrations.

A more typical example is shown in panel B of Figure 4.1, where tricyclic antidepressants were separated. In this example, the retention of all five compounds changes significantly between 5 and 20 % modifier. In the most extreme case, k changed from about 14 at 5 % to about 2 at 20 %. All the curves are nearly parallel over this fairly wide range of modifier concentration. The fact that the curves do not cross each other indicates that no peak reversals occurred, so changing modifier concentration tends to have a large effect on retention but much less effect on selectivity.

4.2 Temperature

Changes in temperature tend to have less effect of retention than modifier concentration, but can sometimes result in significant shifts in selectivity. After the modifier concentration is varied to get all the solutes into a reasonably short retention window, the temperature can be varied to potentially change selectivity.

Chromatograms of two sulfonamides⁷¹ at various temperatures, but with all other variables held constant, show baseline separation with the elution order 1, 2 at 28 °C, but coelution with a decrease in retention at 40 °C, then increasing retention and increasing resolution with elution order 2, 1 above 60 °C as shown in Figure 4.2.

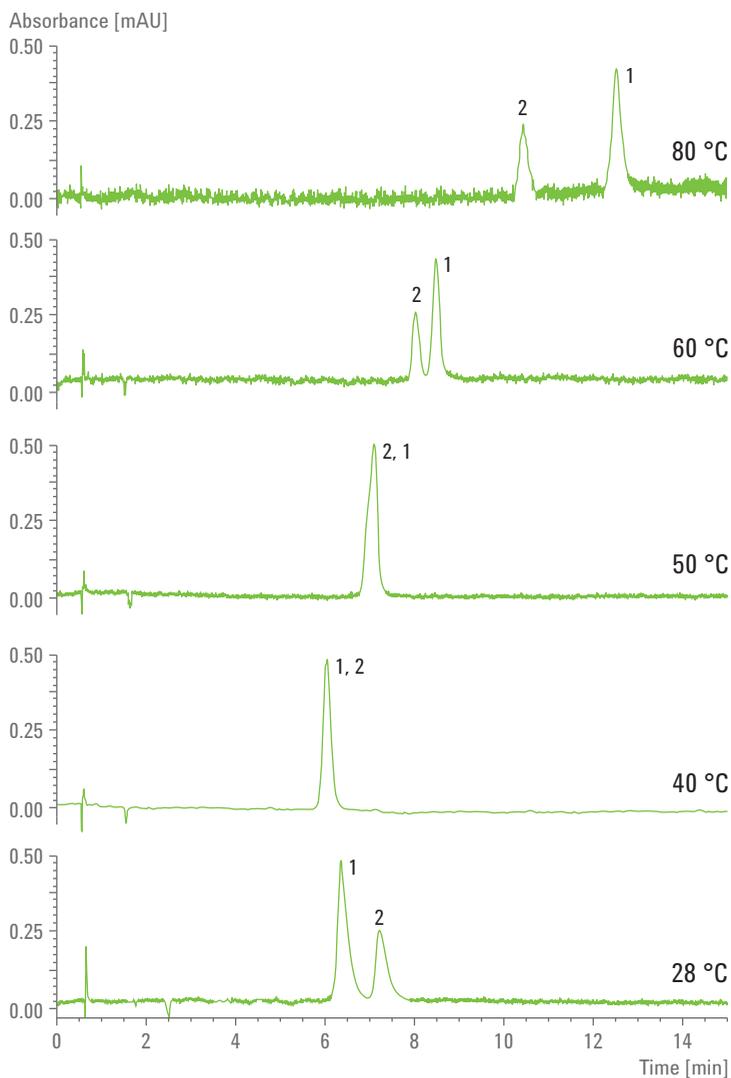


Figure 4.2 Peak reversal of two sulfonamides caused by small changes in temperature.

Three additional graphical examples^{70,72,73} are shown in Figure 4.3. In the first example, panel A, several stimulants were separated at constant flow, mobile phase composition, and column outlet pressure, but the temperature was changed from 30 to 90 °C. The retention of some of the compounds increased, some decreased and some stayed nearly the same when the temperature was increased. Crossing curves indicate peak reversals. Note, however, that the overall change in retention was modest, particularly when compared to the effect of modifier concentration on retention.

Similarly, 10 antidepressants were separated under identical conditions, except for temperature (panel B). Again, some of the compounds increased retention, some decreased, while others hardly changed between 30 and 60 °C. The curves are nearly horizontal, indicating only modest changes in retention, but several cross indicating peak reversals.

A third example, panel C, showing the separation of antipsychotics demonstrates behavior similar to the other two families of compounds. There is a telling detail. At about 6 minutes, three of the solutes coelute at 35 °C, producing a single symmetrical peak. Two other peaks also coelute near 7 minutes. However, at 40 °C, only 5 degrees warmer, the coeluting three peaks near 6 minutes, and the two peaks near 7 minutes were all baseline resolved! These compounds all have the same central three-ring structure, and differ by only a few substitutions on the rings.

Unfortunately, at 40 °C, peaks near 3 minutes and at 8.5 minutes coelute. Raising the temperature further to 45 °C results in both the first triplet and the doublet remaining baseline resolved. In fact, almost all the solutes are nearly evenly spaced and well-resolved, at 45 °C, except for the second and third least retained peaks, which merged at 40 °C and remained coeluting at 45 °C and above. Under a few conditions like this, a temperature program might separate all the compounds.

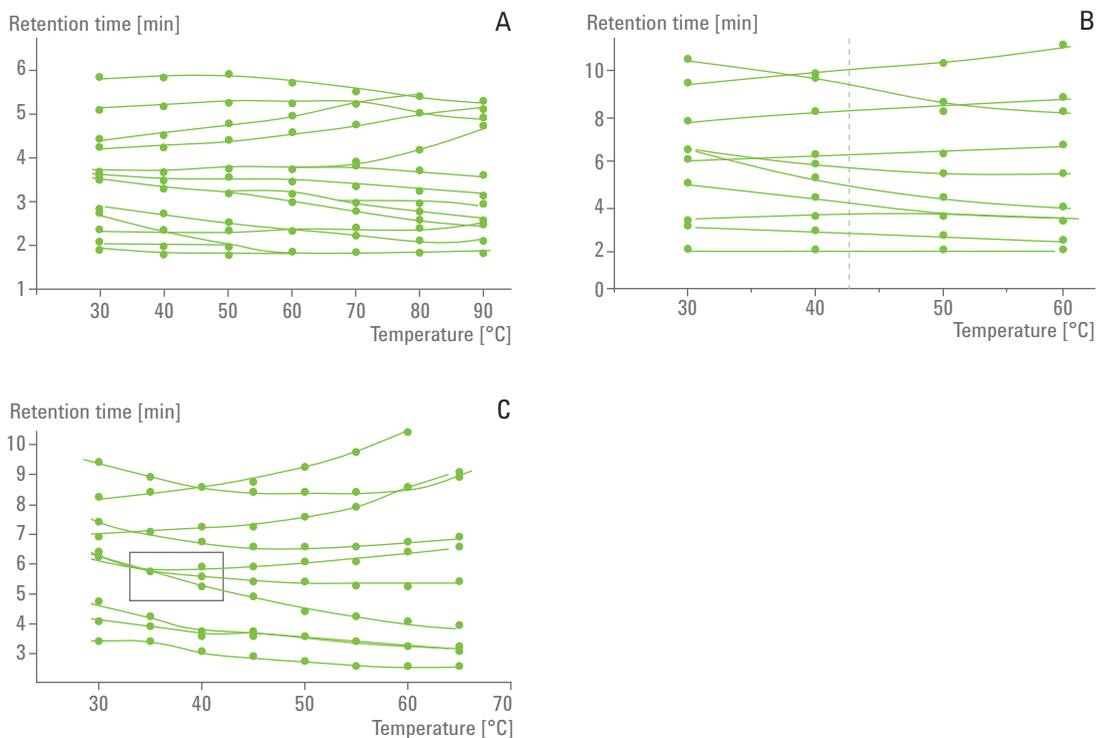


Figure 4.3 Effect of temperature on retention and selectivity. Some compounds increase retention while others decrease retention, causing large changes in selectivity. A: stimulants; B: antidepressants; C: antipsychotics.

4.3 Pressure

With pure CO₂, pressure is a primary control parameter. However, with modified fluids pressure tends to have much less effect on either retention or selectivity, particularly with higher concentrations of more polar modifiers. Again, three different families of compounds^{70,73,74}, including phenylurea pesticides, tricyclic antidepressants and stimulants were separated at various column outlet pressures with the rest of the conditions held constant. The curves, shown in panels A, B and C of Figure 4.4, for the various members of each family are nearly parallel and rather flat, except for the stimulants. There are a few curves that cross but nothing like what occurs when temperature is the variable.

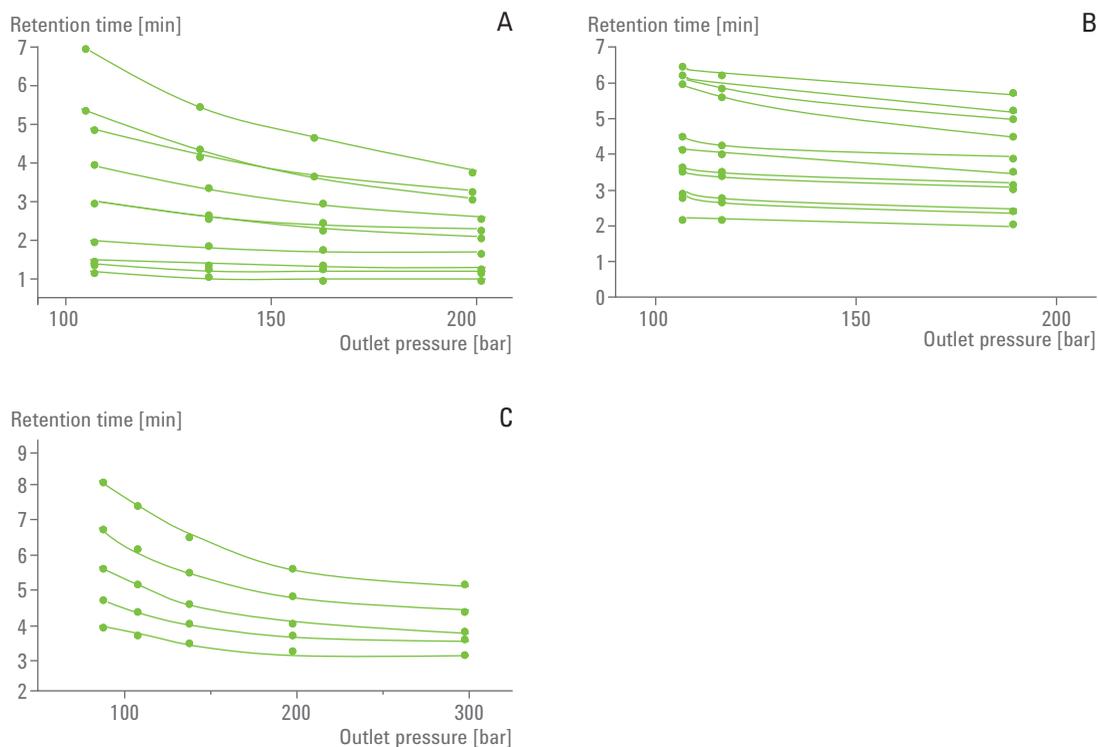


Figure 4.4 Effect of column outlet pressure on retention and selectivity. A: phenylurea herbicides, note the peak reversal between peaks 8 and 9; B: tricyclic antidepressants; C: stimulants.

Translating a method from 5- μm particles to sub-2- μm particles results in a significant increase in column head pressure when using the smaller particles. The pressure drop is proportional to the inverse square of the particle diameter. Thus, at constant efficiency (from a decrease in column length), a 1.8- μm column will have 7.7-times higher pressure drop than a column with 5- μm particles. This means that the average pressure and density of the mobile phase in the two columns will be significantly different. This can have subtle effects on retention and selectivity as outlined above. This is most obvious at low modifier concentrations and especially with low column outlet pressures (less than 120 bar) and temperatures (less than 50 °C), as shown previously for the density of pure CO_2 in Figure 2.1, and for CO_2 and methanol in Figure 2.3. The slopes of the density against pressure curves tend to be less steep at higher pressures, minimizing such effects.

The difference in average density on the two columns has been recently introduced as a *new* controversy, but the concept has been well understood for decades. If the original work on the larger particles was performed well above 100 bar (that is, 150 to 200 bar), the outlet pressure with the smaller particles could be reduced to make the average pressure (and density) on the two columns the same or at least similar.

If a problem occurs in translating a method from a large-particle column to a smaller-particle column, take the pressure drop across the column with the smaller particles and divide by two to yield the average pressure drop. Increase the column outlet pressure of the column with the larger particles until the average pressure across that column is the same as the average pressure across the column with the smaller particles. The density effects are nonlinear, so small additional changes in column outlet pressure may be required to achieve similar selectivity.

Alternately, simply raising the outlet pressure on the column with the larger particles tends to normalize or minimize the effect.

4.3.1

Average column density

It is difficult to calculate the average density in a column with binary fluids. Remember that there are few systems where the density of binary fluids is even marginally known or can be calculated. The best example is methanol/CO₂. There is a database and an equation of state that works over a narrow range of conditions⁷⁵⁻⁷⁷.

While the lack of data may change in the future, past history suggests the generation and collection of such data is unlikely with the present lack of funding for basic research in SFC.

4.4

Flow

In HPLC, changing the flow rate tends to have little effect on selectivity, but can shorten run times provided that resolution can be traded for speed. SFC is a little more complicated, since increasing the flow rate increases the pressure drop both in the column and in the connector tubing. Since the backpressure regulator holds the system outlet pressure constant, the increased pressure drop results in the pump pressure increasing. Higher pressure results in modest increases in density and decreases in retention (lower *k*) beyond that expected due simply to the higher flow rate as outlined in a previous section.

The effect of flow rate on the retention of caffeine, theophylline, theobromine and uracil is shown in Figure 4.5. The pressure drop increased from 33 bar at 2 mL/min to 147 bar at 5 mL/min. Retention decreased about 2.5-times when flow was increased by the same amount. Since the change in retention was proportional to the change in flow rate, the results indicate that the

effect of the pressure drop is minimal. However, normalizing retention (actually k) to the values at 2 mL/min results in the values shown in Figure 4.6. Retention (k) decreased as much as 13 %. While this is a rather mild effect, you should be aware of this form of behavior.

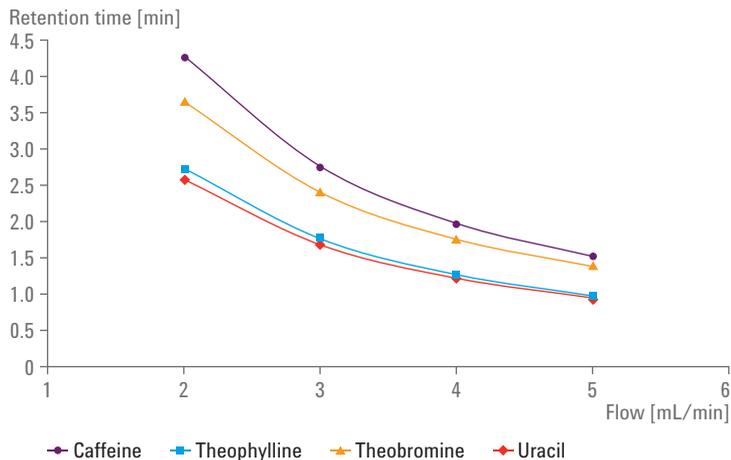


Figure 4.5 Effect of flow rate on the retention of caffeine, theophylline, theobromine and uracil. The curves are nonlinear due to the change in density of the mobile phase, caused by the increased column pressure drop at higher flow rates. The pressure drop increased from 33 to 147 bar (188 to 297-bar inlet pressure). Conditions 15 % methanol at 50 °C and 150-bar outlet pressure. Column: 4.6 by 150 mm, 3.5 μ m RX-SIL.

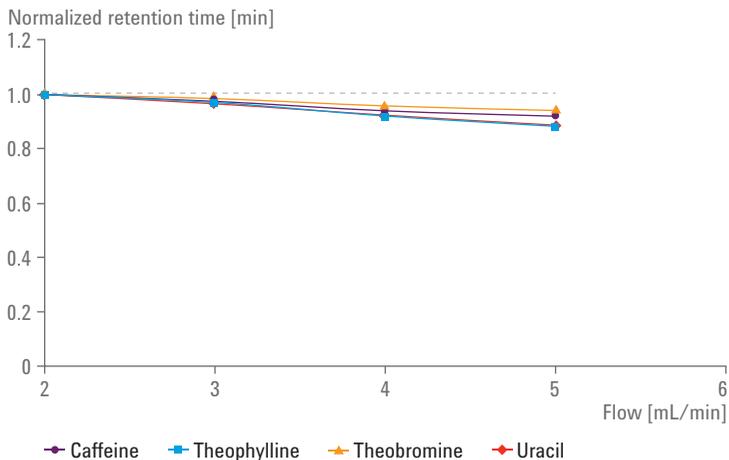


Figure 4.6 Normalized retention times from Figure 4.2 showing that retention decreased as much as 13 % when the flow rate was increased from 2 to 5 mL/min due to the increase in the density of the mobile phase through the column. The pressure drop increased from 33 to 147 bar (188 to 297-bar inlet pressure). Other conditions were held constant at 15 % methanol, 50 °C, and 150-bar outlet pressure. Column: 4.6 by 150 mm, 3.5 μ m, RX-SIL.

This data was collected using a 4.6 by 150-mm column, packed with 3.5- μm RX-SIL particles ($P_{in} = 188$ to 297 bar). Columns packed with sub-2- μm particles are likely to have even larger deviations, due to changes in pressure drops. However, at near optimum flow rates, column pressure drops seldom exceed 250 bar (less than 400-bar outlet pressure) and, at modest modifier concentrations ΔP values are often about 50 to 100 bar, in a similar range to those in Figure 4.4.

4.5 Generalizations on effects of control variables on retention and selectivity

Modifier concentration is the most effective way change the retention of peaks substantially without changing columns. Doubling modifier concentration roughly halves retention. Modifier concentration should be the first variable to use to move peaks into an appropriate retention window.

If peaks are inadequately resolved, a small change in temperature can often improve resolution between specific pairs of peaks. A change of ± 10 degrees usually indicates whether selectivity improves. If it does, try additional increments of 5 to 10 degrees in the best direction, for optimization.

Pressure should be considered as a secondary control variable, but a significant pressure step (that is, 25 to 50 bar) might produce subtle changes in retention or selectivity, or both. Flow rate should be set to near the optimum until resolution can be optimized or maximized for all the solutes. The flow could then be increased until the resolution is just acceptable.

Chapter 1 “Introduction to Supercritical Fluid Chromatography” was intended to give you a broad overview of what SFC is and what SFC is not. Chapter 2 “The Mobile Phase” familiarized you with the various aspects of the mobile phase characteristics such as the low polarity of pure CO₂, the large impact on solvent strength when polar modifiers are added, how the solvent strength is a nonlinear function of concentration, and how many solutes require highly polar additives in the mobile phase to elute. Chapter 3 “The Stationary Phase” explained the size and shape of the appropriate column hardware, as well as commented on the relative polarity and interactions of the actual stationary phases available. Chapter 4 “Effect of Mobile Phase Variables on Retention and Selectivity” described the effect of the various mobile phase variables on retention and selectivity, showing that modifier concentration has, by far, the biggest effect on retention but much less effect on selectivity compared to the other instrumental mobile phase set points. Temperature has much less effect on retention but can have a surprisingly significant effect on selectivity. Pressure is simply a secondary control variable, having only a minor impact on either retention or selectivity. Flow has even less effect on selectivity and only through changing the pressure drop across the column. It is now time to combine all this background information into a rational approach to method development.

5.1 Matching solute and phase polarity

The old adage of *like-dissolves-like*, meaning a solvent with similar functionality to a solute is likely to be a good solvent, while a solvent with different chemical characteristics is likely to be a poor solvent. For example, salt and water compared to salt and heptane. This adage can be extended to chromatography by choosing stationary and mobile phases that have similar chemical characteristics to the solutes of interest. CO₂ in some ways can be viewed as a relatively *inert* diluent, when used to separate relatively polar solutes. The modifiers polarity and chemical functionality primarily dictates the characteristics of the mobile phase. Specifically, there needs to be a strong interaction between the solutes and the stationary phase, and between the solutes and the modifier.

Such strong competing interactions result in rapid equilibration. Weak interactions are ill-defined, and as a consequence, result in long equilibration times and poor retention time reproducibility.

Various solute functionalities are listed across the top of Figure 3.2. Below the solutes are several mobile and stationary phases, with arrows indicating the appropriate ranges of polarities for each phase. These are approximations, but provide good starting points. Finding an appropriate solute family, then dropping a vertical line indicates several possible combinations of mobile and stationary phases likely to produce a viable separation.

Acids and bases will require polar stationary and mobile phases, such as bare silica, diol or amino. Solute that are capable of strong hydrogen bonding should be separated using hydrogen-bonding stationary and mobile phases. Solute that can ionize in aqueous media, depending on pH, will likely need a polar modifier, and an even more polar additive. The additive should be chosen to suppress such potential ionization.

At the other extreme, solute with minimal polar functionality and long hydrocarbon chains, or both, may be best separated with a nonpolar stationary phase such as C18, and a relatively non-polar modifier. For such separations, it is unlikely that an additive will be required.

5.1.1 Additives

In order to choose an additive, another analogy that may be helpful is the *acid-base shake-out test*. Bases partition into aqueous media when in contact with a nonmiscible organic solvent, under conditions where the pH in the aqueous phase allows the base to ionize. However, at pHs in the aqueous phase that suppress ionization, the same base will likely partition out of the aqueous phase into the organic phase. Think of the stationary phase as the aqueous phase, and the mobile phase as the organic phase. The additive should be such that it suppresses ionization of the basic solute, keeping it from adsorbing onto the stationary phase.

True optimization may require trying several different additives. However, most similar additives will produce similar effects, and it is usually not worth the extra effort, except in extreme circumstances where nothing much seems to work.

One of the most obvious cases for the need for an additive involves primary or secondary aliphatic amines. In such cases, it may be worthwhile to start with an ethylpyridine column to attempt to avoid the use of a basic additive, particularly when using a detector like a mass spectrometer. However, ethylpyridine columns tend to be much more retentive than the classic phases (cyano, silica, diol, amino) and often require a relatively higher modifier concentration. This decreases the diffusion coefficients of the solutes, slowing the optimum linear velocity, and increases viscosity, increasing pressure drop. Alternately, a strong base, such as triethylamine, added at about 0.1 % in the modifier, will probably yield full column efficiency while allowing a much lower modifier concentration (with higher speed and efficiency).

Amphoteric compounds are usually best dealt with using an additive such as ammonium formate or ammonium acetate, often supplemented with a much stronger acid or base. Such additives tend to suppress both acidic and basic ionization of such solutes, or form neutral ion pairs.

5.2 Polarity windows

The phases should create a *polarity window* bracketing the solutes. If the sample consists of a broad range of polarity, size and functionality, the difference in polarity of the phases should be wider, creating a wide *window* of polarity that can be narrowed through composition programming, over a wide range of composition. If the sample consists of a family of closely related compounds with similar polarity, the phases should be closer together and similar to the solutes.

5.3 Getting started

It is important to define the goals of the separation at the beginning. Optimization may take different paths if, on the one hand, the goal is the fastest separation with some minimal resolution between peaks, or, on the other, trace analysis. One goal should always be to develop a method wasting as little time as possible. Toward that end, always try to start with high concentrations of appropriate standards and strong elution conditions to avoid as much uncertainty as possible. If a specialty detector is required, such as an evaporative light scattering (ELS) detector, try to separate developing the separation method, and optimizing the detector. The ELS detector is usually used when the solutes have poor chromophores. Use solutes as near as possible to the solutes of interest, but that do have a chromophore. You can then use a UV detector to help optimize the ELS detector using the solute with a chromophore, which should produce operating conditions nearly appropriate for the solutes of interest.

5.4 Polar solutes

The next sections describe a step-by-step process toward developing an optimized method. In Figure 5.1 shows a decision tree for polar solutes that follows these guidelines.

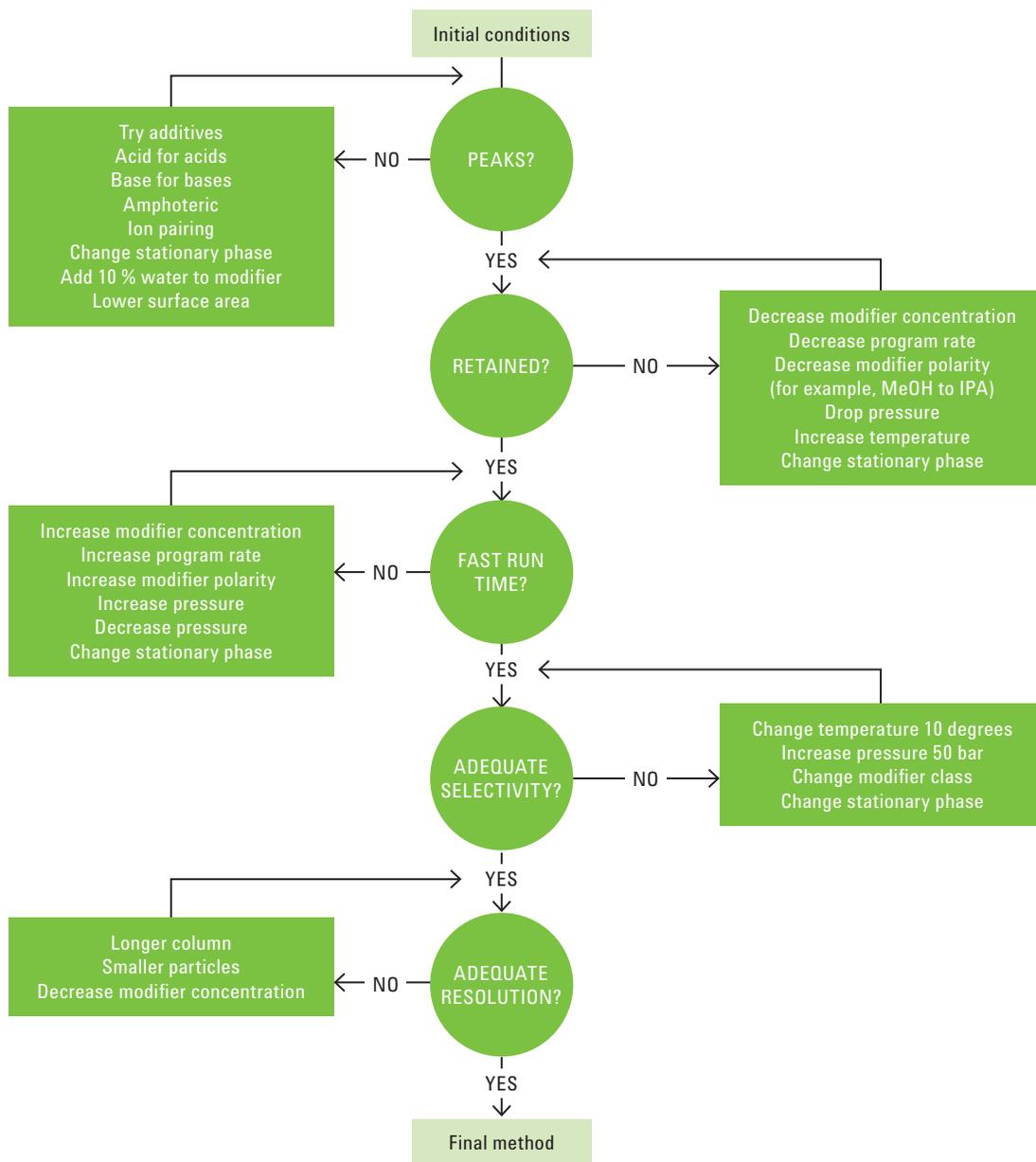


Figure 5.1 Decision tree for method development using polar solutes.

Highly polar solutes such as polyfunctional acids or aliphatic amines, are found in the upper right of Figure 3.2. Dropping a vertical line indicates that any of the polar stationary phases could be used. The line also suggests methanol and an appropriate additive will probably be required.

5.4.1 Is an additive needed?

The first question to be addressed with polar solutes is whether the mobile phase needs to include an additive. Choose a polar stationary phase such as bare silica. Use 40 % methanol in CO₂ at 50 °C, 150-bar outlet pressure at 1.5-times the optimum flow rate. Most solutes should elute within a few minutes under these strong conditions. After the first injection, are there symmetrical, high-efficiency peaks?

5.4.1.1 Peaks?

If there are appropriately shaped peaks, without tailing, no additive is needed and you should proceed to optimize the modifier concentration.

5.4.1.2 No peaks?

If nothing elutes or peaks tail badly, an additive is needed. Usually 0.1 to 0.2 % added to the modifier is adequate. If the solutes are bases, add a strong base such as ammonia or triethylamine. If the solutes are acids, add a strong acid such as trifluoroacetic acid. Many solutes are multifunctional. For such solutes, an amphoteric additive such as ammonium formate or ammonium acetate may be appropriate. On the second injection, with additive included, do symmetrical, high-efficiency peaks emerge?

5.4.1.3 Peaks!

In more than 90 % of the cases, the addition of an additive produces appropriately shaped peaks. If, with the additive, peaks emerge, proceed to optimizing the modifier concentration in exactly the same way as if no additive were required. It only took a few fast isocratic runs to answer one of the more important questions about polar solutes.

5.4.1.4 Still no peaks?

If no peaks emerge or those that do have poor peak shapes, try ion pairing (acidic additive for basic solutes, and vice versa). If still no peaks emerge or peak shapes are poor, the success or failure of the separation will depend on finding the right additive or combination of additives. The last thing to try is to add 10 % water to the methanol, along with additives.

An alternative is to switch to one of the newer phases such as ethylpyridine for bases, or *Premier* for acids. These stationary phases may enable the elution of the solutes even without additives.

5.4.2 Optimizing modifier concentration – methanol

Methanol is one of the strongest solvents that is completely miscible with CO₂. As such it is often too strong a solvent for many solutes, causing them to coelute near the column hold-up time, at 40 % methanol.

Using the rule of thumb that halving modifier concentration roughly doubles retention times, progressively decrease the modifier concentration by a factor of two (to 20 %, then 10 %, then 5 %) and observe the retention times of all the peaks. By slowly changing the concentration of modifier, it is fairly clear if peaks reverse elution order. On the other hand, it only takes a few isocratic runs to characterize what further steps might be necessary.

If the solutes display a wide range of retention, a composition gradient is in order. If the peaks are significantly retained but tend to bunch together with similar retention times, an isocratic method is probably in order. Develop either, such that the run time is reasonably short, but do not expect to adequately resolve all the peaks using modifier concentration alone. When the modifier composition is near optimum and all the peaks are at least partially resolved, proceed to using the column temperature to modify selectivity.

5.4.2.1 Methanol still too strong

If, at 5 % methanol, some of the peaks are still nearly unretained with inadequate resolution, switch to a weaker solvent such as either ethanol or isopropanol. In most cases, one of the alcohols will produce a viable separation. In extreme cases, switching to a different solvent family may be appropriate. If the alcohols are inappropriate, the initial evaluation of the sample was probably wrong.

5.4.3 Using temperature to change selectivity

As pointed out in Chapter 4 “Effect of Mobile Phase Variables on Retention and Selectivity”, temperature tends to have only modest effect on retention but can have significant effect on selectivity. Once the modifier concentration has been optimized to produce a reasonably short run time, changing the temperature by ± 5 to 10 degrees often changes the relative retention of difficult pairs of peaks. If such a change in temperature improves the resolution of difficult pairs, try a larger change in temperature in the same direction.

5.4.4 Pressure

With polar solutes, pressure is a secondary control variable and is unlikely to change either retention or selectivity significantly, particularly at higher modifier concentrations. However, it is always worth changing the pressure in 25 or 50-bar increments, if a separation is not optimized. It is simple to try, takes little time, and may actually be useful. Changing

the outlet pressure changes the density of the fluid, which can have some desirable effects. UV detector noise tends to be lower at higher pressures. Therefore, it is usually advisable to perform trace analysis at higher pressures.

5.5 Low polarity solutes

Many low polarity solutes are amenable to SFC. Many are complex natural products, or homologous series. A few typical lower polarity solute families are listed on the top left of Figure 3.2. Choosing one of the solute types and dropping a vertical line indicates appropriate phases. For compounds with long hydrocarbon chains, the most appropriate stationary phase is probably C18, but others such as C8, phenyl and cyano, may also be useful. The polar stationary phases are likely to produce little retention and distorted peaks, since there will be weak interactions between the polar stationary phases and the nonpolar solutes.

The use of pure CO₂ is unlikely to produce good peak shapes at low temperatures and pressures. The most reliable way to develop a robust method for nonpolar but heavy solutes is to use a modified fluid either isocratically or with composition programming. Gradients are still programmed from low to high modifier concentrations.

A relatively lower polarity modifier such as isopropanol is a good starting point. Since low polarity solutes tend to not hydrogen bond, a nonalcohol for modifier may be better. It is more common to use acetonitrile with lower polarity solutes, compared to higher polarity solutes. Chlorinated solvents are avoided, although they can be used. With such solutes, it is unlikely that an additive would be needed.

5.5.1 Optimizing modifier concentration

With complex samples, it is likely that a gradient will be needed. However, it is always good to minimize confusion by trying a quick run with a high isocratic modifier concentration to make sure that everything elutes. As with the polar solutes, start with 40 % modifier, 50 °C and 150 bar.

It is highly likely that everything will elute rapidly. Halving modifier concentration should double retention. If 5 % isopropanol is still too strong a solvent, 10 % isopropanol can be added to heptane and the mixture used as the modifier.

5.5.2

Temperature and pressure

With polar solutes, small changes in temperature can have significant impact on polar-polar interactions between the solutes and the phases, causing selectivity changes. With much less polar solutes, interactions are likely to be dominated by Van der Waals forces and temperature tends to have less impact on selectivity.

The biggest effect of temperature and pressure will probably be through changing the density of the mobile phase. Retention is likely to increase at higher temperature and lower pressures (lower densities). Retention is also likely to be more a function of density, especially at low concentrations of a lower polarity modifier.

5.6

Multivariate methods

The method development approach outlined above probably seems old-fashioned since it follows a univariate approach. Further, you need to make some near real-time decisions, or waste time and materials. As will be seen with the optimized separations in later chapters, multivariate methods will have a difficult time finding the optimum conditions for many separations. Such multivariate approaches are *much* less likely to produce an optimized separation, compared to a more detailed univariate method development optimization. This is true with changes in temperature, which often causes peak reversals. However, the high speed of SFC makes it possible to rapidly develop such methods, in a much shorter time compared to HPLC.

In this chapter, several case studies are presented on how specific methods were developed. The case studies chosen were all rather difficult, with unusual peak reversals, with odd responses to changes in mobile phase parameters. It is hoped that the straightforward resolution of these difficult mixtures, by following the recommendations in the previous chapters of this monograph, will help you in developing your own methods. It should be stressed that these examples are not typical, and do not represent the norm. Most separations are much easier to optimize, for the specific analytical requirement. You should also remember that there are different criteria for what represents an *optimized* separation. All separations are a trade-off between speed, resolution and sensitivity. In the following examples, all these issues are addressed.

6.1 Case Study 1 – A typical low-polarity sample

This first example is fairly atypical of the sorts of samples commonly separated using SFC over the last 20 years. However, such applications were much more common in the earlier days of SFC. This application hints at the great potential for the technique in fields other than pharmaceutical research.

The separation of paprika oleoresin is a good example of a relatively nonpolar family of compounds⁷⁸. This commercial product is an extract from paprika pepper, which in recent years has replaced most artificial red dyes in processed foods, and is important economically.

There are only two primary colored pigments present: capsanthin, and capsorubin. Each has two hydroxyl groups, one on a ring at each end of a terpenoid chain, as shown in Figure 6.1. These hydroxyls form a large number of mono- and di-esters with a range of fatty acids. Samples tend to contain about 70 compounds, mostly the di-esters. The esters do not significantly change the color of the parent compound, but may help to stabilize it.

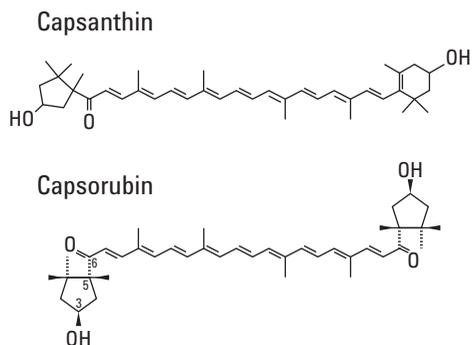


Figure 6.1 The red color found in paprika comes directly from two compounds capsanthin, and capsorubin. They are mostly present as mono- and di-esters formed with various fatty acids. Only a relatively few more polar mono- and di-alcohols exist in the peppers.

These esters are nonpolar, due to the long hydrocarbon tails on the fatty acids. You can find esters at the top and to the left of Figure 6.2. The large vertical arrow indicates appropriate mobile and stationary phases. The structure of the solutes suggests the use of a similar long hydrocarbon tail for the stationary phase, such as on a C18 phase, along with a relatively nonpolar modifier in CO_2 such as isopropanol. Three different C18 columns with slightly different selectivity (Agilent) were used, each with 1.8- μm particles.

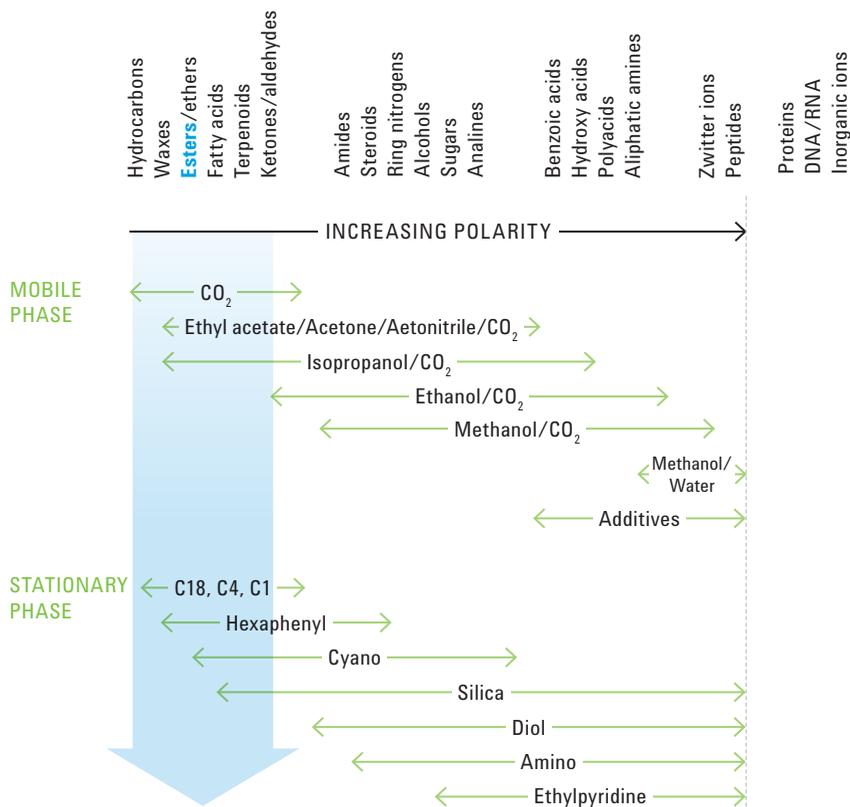


Figure 6.2 Paprika oleoresin contains a large number of mono- and di-esters of capsanthin and capsorubin. The arrow indicates appropriate stationary and mobile phases for esters.

Separations of the same sample using an identical gradient of 5 to 20 % isopropanol in CO_2 on the three C18 columns produced similar but not identical chromatograms with subtle differences in resolution, as shown in Figure 6.3. The peaks were well retained and reasonably spaced. In contrast, the same sample separated on more polar stationary phases, but using the identical gradient and conditions produced mostly poor peak shapes with retention shifted to shorter times, as shown in Figure 6.4. Clearly the interactions between the solutes and these more polar stationary phases are weaker than the interactions with the C18 phases.

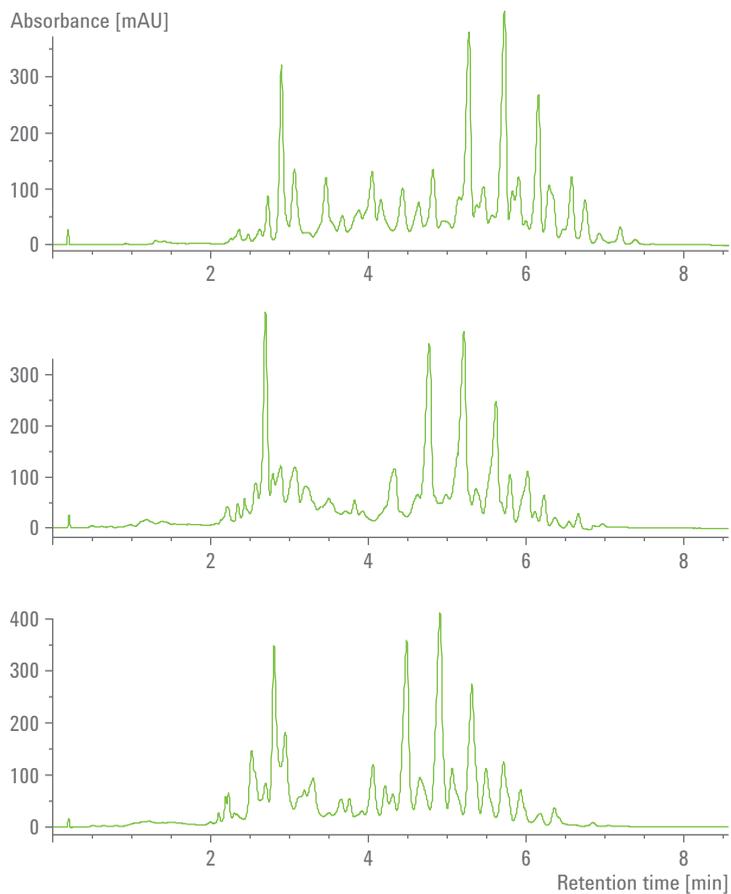


Figure 6.3 Separation of paprika oleoresin on three different C18 columns, all 3 by 100 mm, 1.8 μ m (all from Agilent), using a gradient of 5 to 20 % IPA in 6.5 minutes.

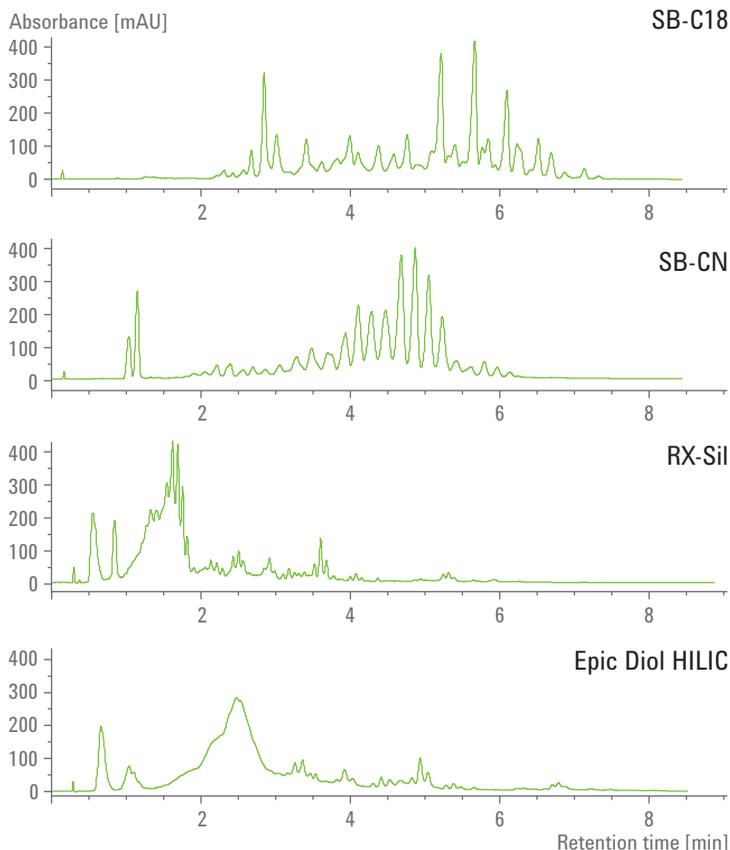


Figure 6.4 The same paprika oleoresin and condition but separated on more polar columns, compared to an SB-C18 column at the top. All the columns were 3 by 100 mm, 1.8 μ m. The nonpolar analytes are poorly retained, particularly on the bare silica and diol phases.

Other modifiers were substituted for isopropanol, using the same gradient and conditions with a ZORBAX SB-C18 column, as shown in Figure 6.5. Acetonitrile, and acetone, are more polar, while ethyl acetate is equally polar compared to isopropanol, according to Snyder's P' scale outlined in Table 2.1, and all three produced shorter retention of the sample. The observed retention order was; acetonitrile (6.2), ethyl acetate (4.3), acetone (5.4), isopropanol (4.3). The numbers in parenthesis are the P' solvent strength values of each solvent from the table. Obviously, the P' values do not correctly predict the retention order for this type of sample. The Hildebrand scale was even worse with the retention order; acetonitrile (0.5), ethyl acetate (0.38–0.48), acetone (0.47–0.53), isopropanol (0.62).

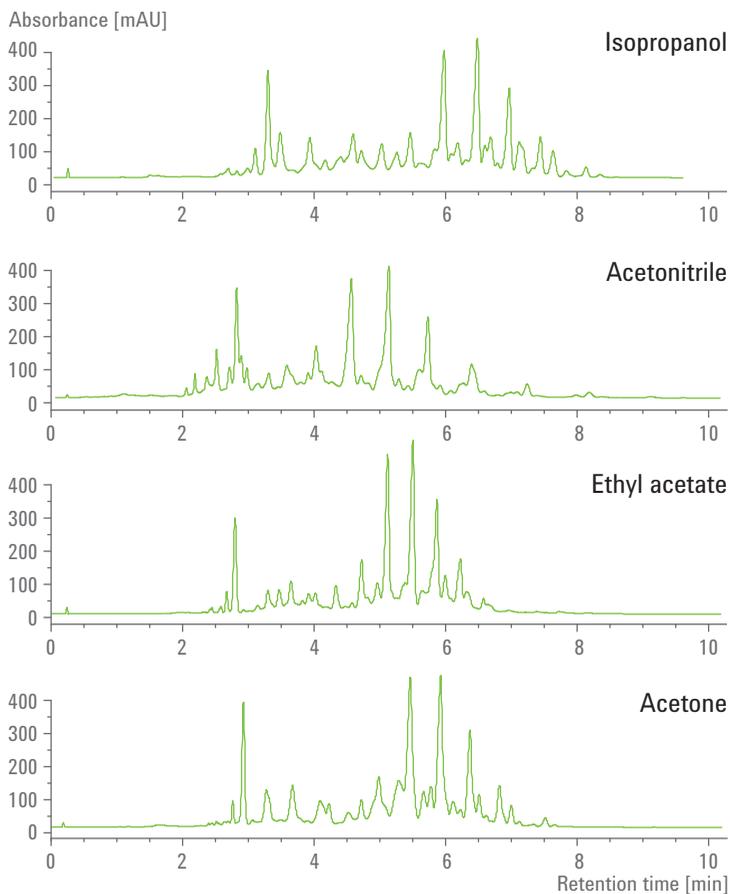


Figure 6.5 Separation of the same paprika oleoresin with the same conditions except substituting other modifiers. All the higher polarity modifiers produced shorter retention than the more polar IPA.

In some ways, this separation resembles reversed phase HPLC. There is greater retention on the less polar stationary phases, and the more polar modifiers decrease retention (more or less). However, in all cases a gradient of increasing concentration of the polar modifier in the less polar CO_2 was used. Thus, the mobile phase polarity increased throughout the gradient, in contrast to reversed phase HPLC, where the mobile phase polarity decreases throughout a gradient.

6.2

Case study 2 – A moderately polar sample

Most separations performed by SFC over the last decade have not been performed with relatively nonpolar solutes and C18 columns, such as in the previous example. Instead, most of the work with SFC has been done on small drug-like molecules.

While the generalizations outlined in Chapter 4 “Effect of Mobile Phase Variables on Retention and Selectivity” are a good guide as to what to expect, they are only generalizations. Caffeine, theophylline and theobromine have often been used as test solutes in SFC since they are relatively safe and require no additive in the mobile phase. They all have the same backbone with four-ring nitrogens. They differ from one another by the position of single methyl substitutions on the nitrogens, as shown in Figure 6.6. Since they are similar in structure, they are good test probes for testing the generalizations. Uracil was added since it has a relatively different structure and may behave differently to changes in conditions. The initial goal was to develop the fastest separation with a resolution greater than three between all the pairs.

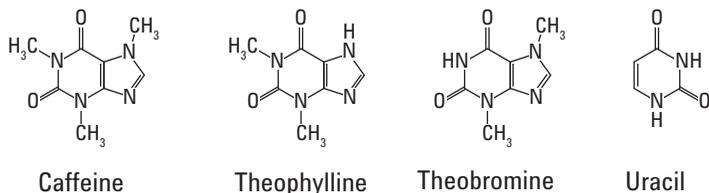


Figure 6.6 Structures of caffeine, theophylline theobromine and uracil.

The solutes were all moderately polar, requiring more polar mobile phase and stationary phases, compared to the previous example. The nitrogens are only present in the rings and unlikely to require an additive. Compounds containing conjugated ring nitrogens are located approximately in the middle of the polarity scale, at the top of Figure 6.7. A bare silica column, and methanol as modifier were selected from the options available. The column used for the experiments was 4.6 by 150 mm, packed with 3.5- μ m RX-SiL. Silica usually yields high efficiency, with minimal complications.

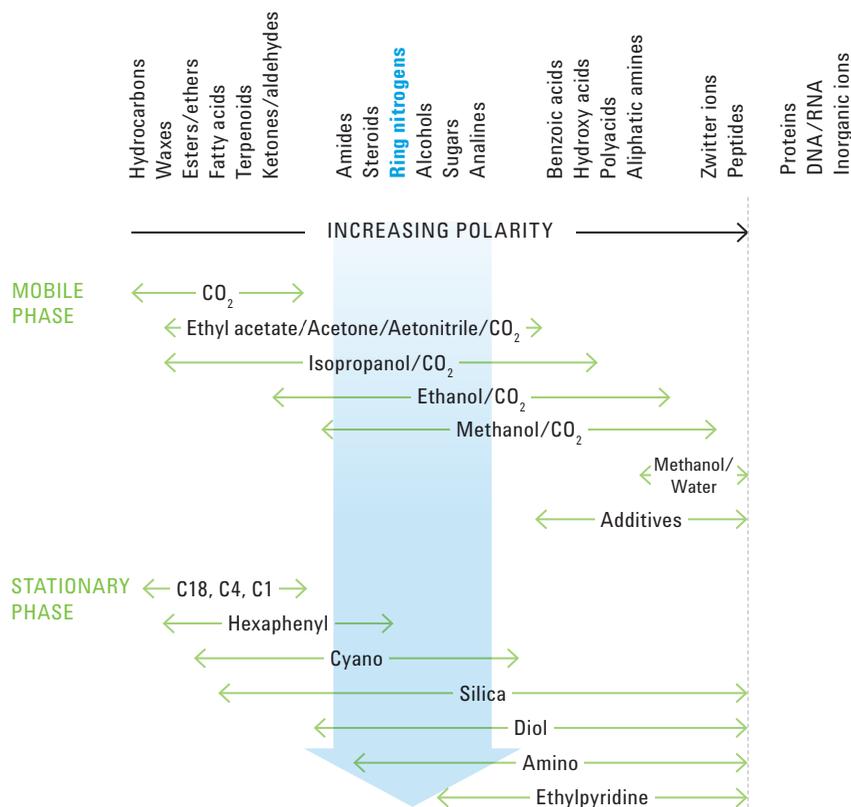


Figure 6.7 The nitrogens in the xanthenes and uracil are all in rings, making them only moderately polar. They are unlikely to require an additive.

Methanol is the most polar modifier that is completely miscible with CO_2 . If there is any question about whether a solute will elute, the obvious first choice for modifier is methanol. Further, it is inexpensive, only moderately toxic, and has low viscosity, as outlined previously.

A generic temperature (50 °C) and pressure (150 bar) were also arbitrarily chosen. The flow was set to about 1.5-times optimum (at 4 mL/min) to speed up method development.

6.2.1

Fastest chromatogram with resolution greater than 3

As outlined previously, the modifier concentration is the most important mobile phase parameter for changing retention. Therefore, the first variable evaluated was the methanol concentration. The first run was made using a high isocratic concentration of methanol, without additive, primarily to see if an additive was needed. If peaks did not elute or eluted with a poor peak shape, an additive would be required. In this case, 30 % methanol eluted all the peaks with high efficiency in a short time (about 1.25 minutes), so no additive was needed. However, one of the peaks was a shoulder on another, as shown in the top chromatogram of Figure 6.8, indicating the modifier concentration should be lowered.

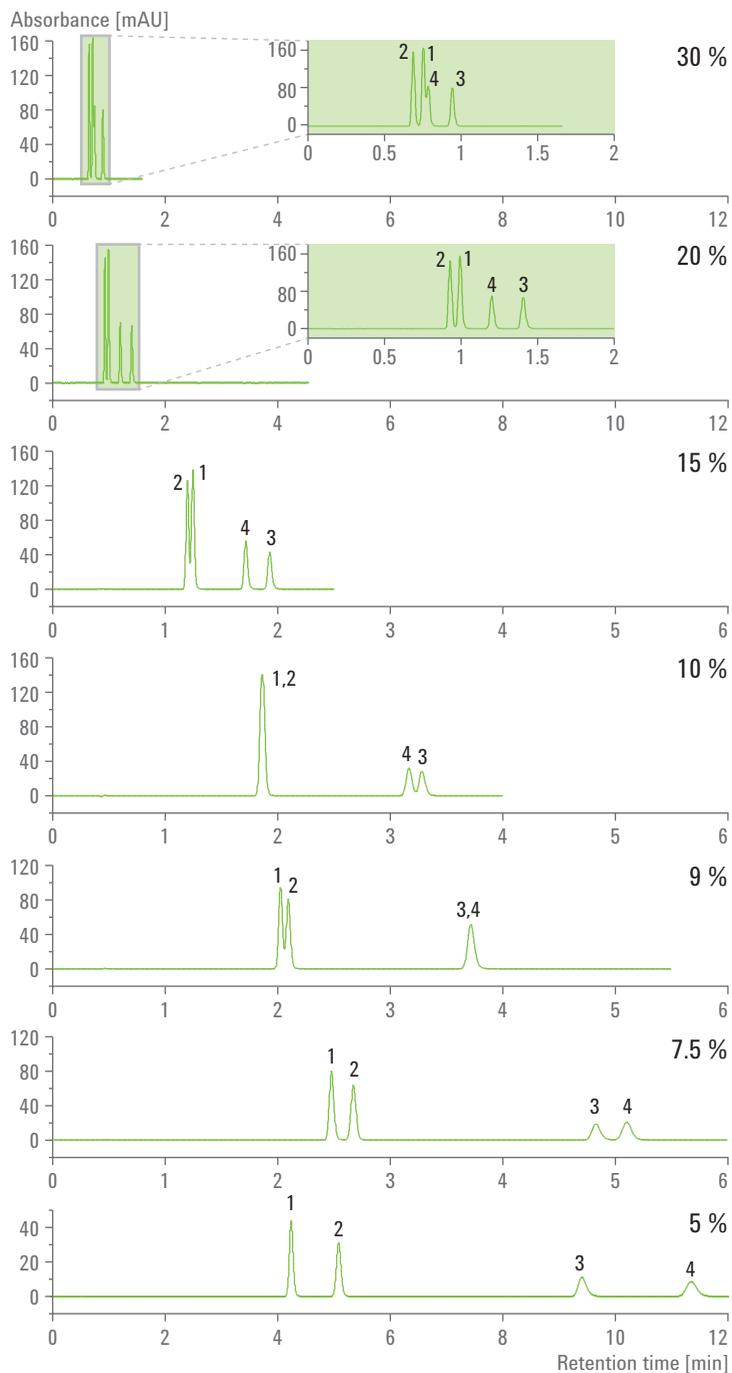


Figure 6.8 The effect of modifier concentration on the separation of caffeine, theophylline, theobromine and uracil at 4 mL/min, 50 °C, 150-bar outlet pressure. , RX-SIL column, 4.6 by 150 mm, 3.5 μ m. Note the unusual multiple peak reversals.

Progressively decreasing the modifier concentration resulted initially in slightly better resolution (at 20 %), but then a loss in baseline resolution between the first two peaks (at 15 %), followed by coelution of the first pair (at 10 %), with loss of resolution between the last pair. This was followed (9 %) by a peak reversal of the first pair, and coelution of the last pair. At lower concentrations (7.5 and 5 %, the resolution within both the first and last pairs progressively increased. All the chromatograms in Figure 6.8 had a time base of 6 minutes, except at 5 % where the time base was 12 minutes.

Superficially, the separations at the lowest modifier concentration (5 %) appeared to give the maximum resolution and could form the basis of further optimization. However, if the goal of the separation was the fastest separation, the time between the two pairs was excessive. The chromatogram collected with 7.5 % methanol was only half as long while maintaining better than baseline resolution. Therefore, 7.5 % methanol at 4 mL/min, 50 °C and 150-bar outlet pressure was initially chosen for further optimization.

Generally, changing temperature tends to have only modest effect on retention, but can often cause significant changes in selectivity. Attempts to improve resolution through changing selectivity, by changing column temperature were unsuccessful in this case, as shown in Figure 6.9. Increasing the temperature from 50 to 60 °C resulted in improved resolution, but only at significantly longer retention times. At 10-degrees lower temperature (40 °C), the baseline resolution of both the first and last pairs was lost. Decreasing temperature a further 10 to 30 degrees resulted in both pairs coeluting as shown in the top chromatogram. Clearly, the different compounds are responding differently to these small changes in temperature. Selectivity is changing, but, in this case, not in a helpful way for method development.

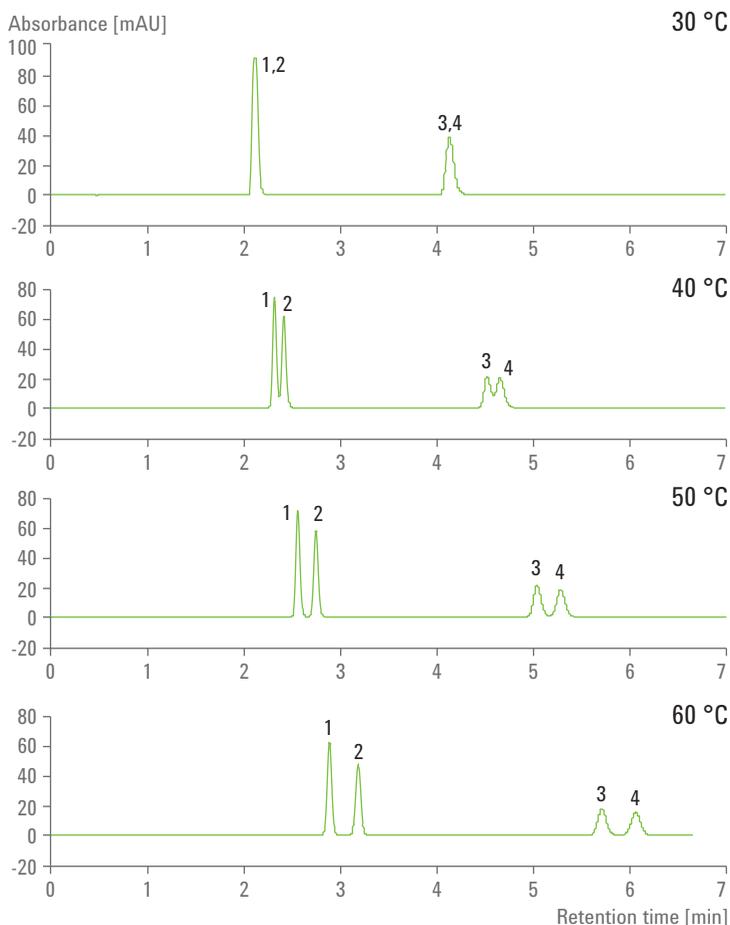


Figure 6.9 The effect of temperature on retention and selectivity for caffeine, theophylline, theobromine and uracil, at 4 mL/min of 7.5 % MeOH, 150-bar outlet pressure.

Pressure is considered to be a secondary control variable, but can have modest effects on retention and selectivity. Returning to 50 °C and 150 bar, the column outlet pressure was varied to observe the effect of column outlet pressure on retention and selectivity. At progressively lower outlet pressures, resolution degraded. At 120 bar, the last two peaks coeluted, but at 100 bar they reversed elution order, as shown in Figure 6.10.

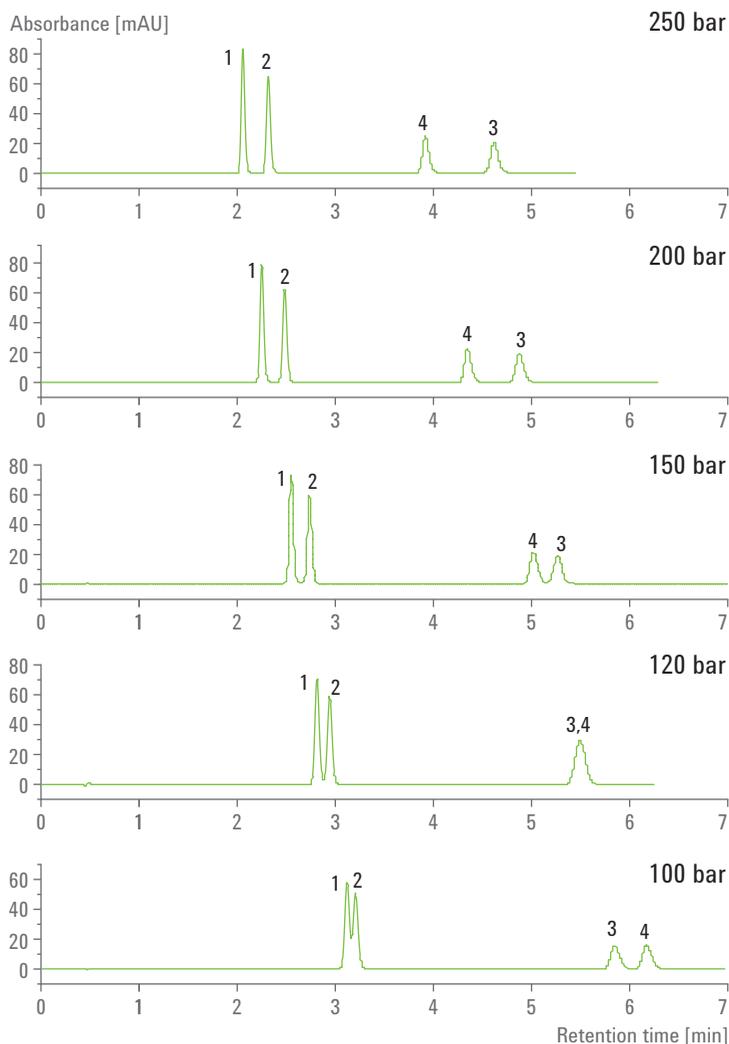


Figure 6.10 Effect of pressure on the retention and selectivity of caffeine, theophylline, theobromine and uracil. Conditions: 4 mL/min of 7.5 % MeOH at 50 °C.

At higher outlet pressures, resolution within the two pairs improved significantly, while retention decreased; both desirable characteristics. The result using 4 mL/min of 7.5 % methanol at 50 °C and 250 bar appeared to be near optimal with a run time of about 5 minutes.

This is an unsatisfactory optimization since there is still a rather large time gap between the second and third eluting peaks. Re-evaluating Figure 6.8, notice that at 20 % methanol, all the peaks were nearly baseline resolved in just over 1.25 minutes. This is substantially faster

than the optimized result with 7.5 % methanol. However, this set of conditions was not initially selected for further optimization, since it is fairly obvious that subtle changes in modifier concentration are unlikely to result in significant improvement in resolution. This is because the results at both lower (15 %) and higher (30 %) concentrations produce worse resolution due to the reversal in elution order of two of the peaks over this range. Thus, you must rely on temperature and pressure to try to further improve the separation, using this starting point.

It was found that when using this higher modifier concentration (20 %), the effect of both outlet pressure and temperature changed, probably because the modifier concentration becomes even more dominant over the density of the mobile phase. Resolution was improved by decreasing the column outlet pressure (not shown). Decreasing flow rate also improved resolution, particularly of the first pair, probably by decreasing the pressure drop and density gradient across the column.

Surprisingly, temperature had almost no effect on either retention or selectivity under these conditions. Chromatograms collected at 2 mL/min of 20 % methanol at 100-bar outlet pressure, between 30 and 60 °C, are presented in Figure 6.11. Compared to Figure 6.9, they show remarkably little change in either retention or selectivity between 30 and 60 °C. Overall resolution is better, with a run time reduced to 3.25 minutes. The optimum temperature was the lowest tried, whereas the previous optimization found the highest temperature was best. Similarly the lowest pressure gave the best separation whereas, in the previous optimization, the highest pressure gave the best result.

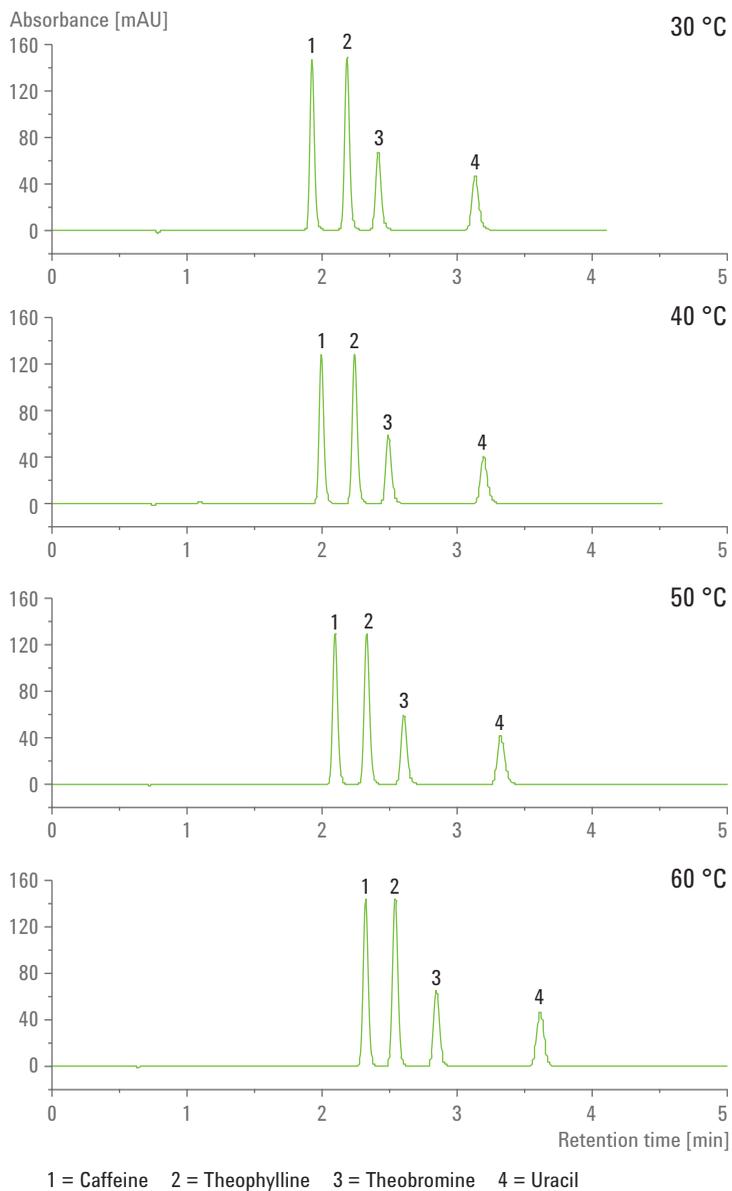


Figure 6.11 The effect of temperature at 2 mL/min of 20 % MeOH and 100-bar outlet pressure. Compared to Figure 6.9, the lack of change in selectivity is surprising.

A few minor adjustments of conditions produced a separation optimized for speed with *resolution* greater than 3, with a run time of less than 2 minutes, using 3.5 mL/min of 20 % methanol at 60 °C and 100 bar, as shown in Figure 6.12.

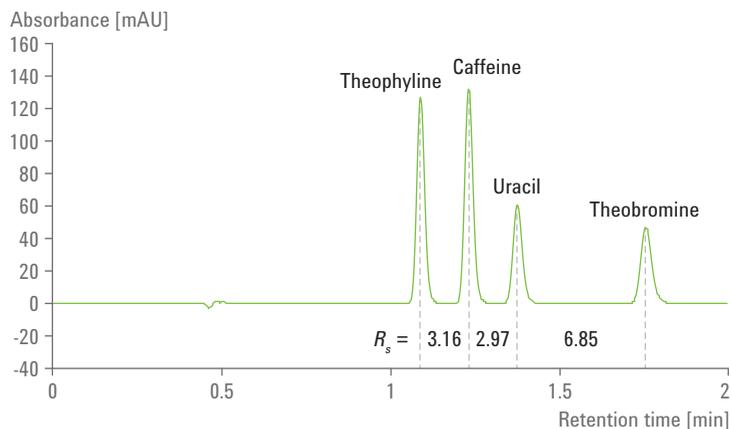


Figure 6.12 Fastest separation with resolution near 3 for all pairs. The numbers under the peaks are the resolutions between the peaks. Conditions: 3.5 mL/min of 20 % MeOH, 30 °C and 100 bar.

6.2.2 Trace analysis

Since all separations involve a trade-off between speed, resolution, and sensitivity, it is important to define the objective of the analysis. The fastest separation with resolution about or greater than 3 was obtained at 3.5 mL/min with 20 % methanol in CO₂ at 30 °C and 100 bar, as shown in Figure 6.12. However, if the goal is trace analysis, the primary requirement is the highest sensitivity with adequate resolution, making speed a secondary consideration.

The minimum resolution for trace analysis is usually considered to be greater than 6. UV noise is typically highest at the lowest pressures. The chromatogram in Figure 6.12 was collected at the lowest pressure, with the highest noise. Further, from the method development experience, it appears that it is unlikely resolution can be increased to greater than 6 from this starting point. These two facts suggest that the chromatogram with the highest speed is unlikely to be the best for trace analysis.

Another standard of the same solutes was prepared, in which the second peak was at the trace level while the first peak was a major component, to demonstrate a typical scenario in trace analysis. In this situation, the minor component is likely to be in the tail of the main component (tailing is failure to properly return to the baseline, as with a Gaussian peak). The potential presence of a tail is the basis for requiring such high resolution for trace analysis. So far, the greatest resolution between the first two peaks was obtained using 5 % methanol, at 50 °C and 250 bar, as seen in the lower chromatogram in Figure 6.8. These conditions were used as the starting point for optimization for trace analysis. Decreasing the modifier

concentration increases retention time, but also increases resolution, which is in the desired direction. It was also found that a slight increase in temperature to 60 °C, further increased resolution between the first two peaks. The best separation for trace analysis, related to the first two peaks, was found at 5.5 % methanol at 4 mL/min, 60 °C and 250-bar outlet pressure, as shown in Figure 6.13.

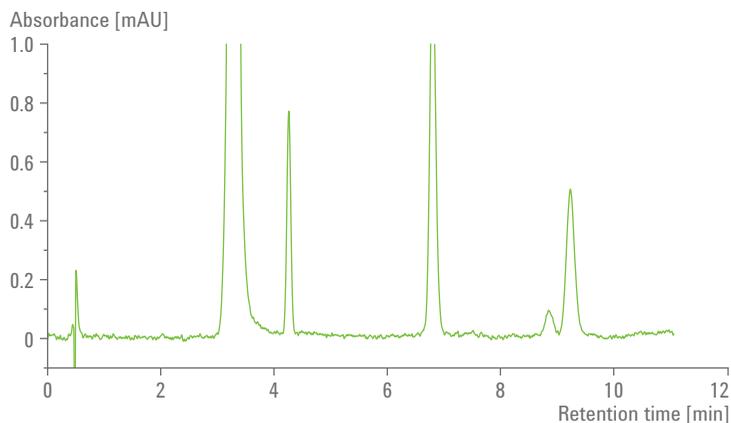


Figure 6.13 Optimization for trace analysis. The resolution between the first 2 peaks was increased to 8.68. The first peak is about 800 mAU while the second trace peak is about 0.8 mAU. Thus, the smaller peak is about 0.1 % of the larger peak, and has an S/N of about 60–80. The LOQ with S/N greater than 10 is estimated at 0.013–0.017 %. Conditions: 4 mL/min, 5.5 % MeOH, 60 °C, 250 bar.

The primary component was the first peak, which was about 800 mAU high. The second peak elutes in the tail of the main peak, which is usually undesirable. However, the resolution between the main peak and the following trace component was 8.68, which enables easy quantification. The trace component is about 0.8 mAU high or 0.1 % of the main peak yet it has signal-to-noise between 60 and 80. The limit of quantification (with signal-to-noise greater than 10) is estimated at 0.013 to 0.017 % of the main component, which is nearly an order of magnitude better than typically required for trace analysis.

In this case, resolution between the first two peaks is probably excessive to requirements. Increasing the initial modifier concentration slightly should decrease resolution from 8.68 to closer to 6, while decreasing run time. Further, after the critical pair elute, the composition could then be programmed to higher values, to more rapidly elute the well-resolved other pair.

Separations can be optimized in several ways. The final conditions depend very much on where you start. At low modifier concentration, changes in both temperature and pressure created multiple peak reversals. At higher modifier concentrations, no peak reversals were observed with changes in temperature or pressure. Programming from low to high modifier concentrations at any fixed temperature and outlet pressure is likely to result in mixed results (variable peak reversals) depending on the rate of programming. The number of peak reversals, observed with these solutes, is rather unusual, but it is important for the user to understand what is possible. Most separations are much more straightforward.

6.3

Case study 3 – Sulfonamides

The sulfonamides are an older family of antibiotics, still widely used in humans. However, most are used today in veterinary medicine. Many different sulfonamides continue to be used, but they have been banned, as a class, as residues in food products. The structures of nine such compounds are presented in Figure 6.14. While one side of all these compounds is identical, the other side contains a wide range of functionalities.

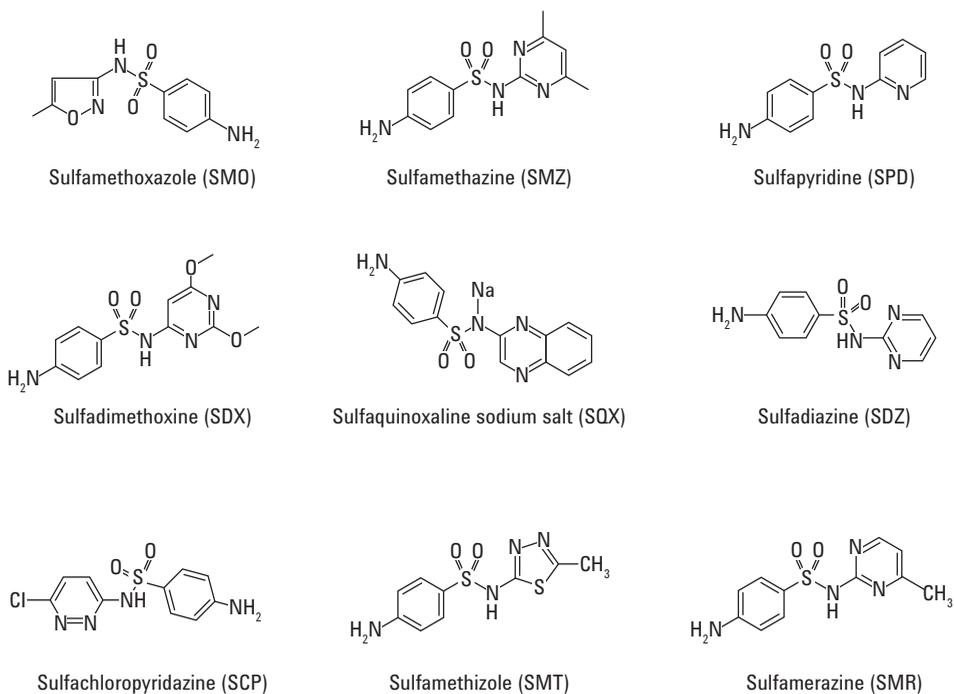


Figure 6.14 Structures of the nine sulfonamides.

This was a challenging sample. The polarity of these compounds is modest, as suggested by Figure 6.15, and again, may not require an additive. However, the similarity of the molecules has traditionally made them difficult to separate. The minimum acceptable resolution between any two peaks should always be greater than 1.5, which became the de facto goal of this method development.

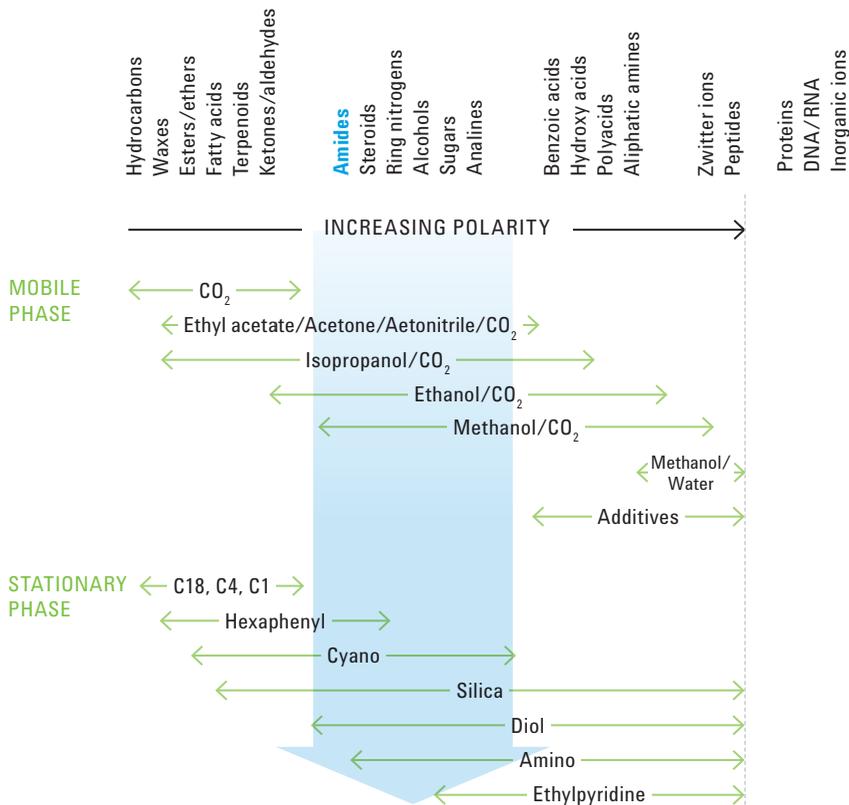


Figure 6.15 Relative polarity of sulfonamides showing options for mobile and stationary phases.

Initial conditions⁷⁹ for the separation of these nine similar compounds were: 2 mL/min of methanol modified CO_2 , with a gradient from 5 to 30 %, at 50 °C and 150 bar, using a 3 by 100 mm, 1.8- μm RX-SIL column. Preliminary experiments showed that all the compounds in the mix eluted at fairly modest methanol concentrations, but several coeluted. Peak shapes were reasonable, so no additive was required. Peaks for several of the compounds fronted slightly. Changing the sample solvent from methanol to isopropanol significantly improved peak shapes.

All the compounds eluted, but closely spaced together, and responded differently to changes in temperature and pressure. Changing one parameter tended to improve the resolution between some pairs of peaks, but degraded the resolution between others. An alternate form of data presentation was used here, where the resolution between the four most difficult pairs of peaks was plotted vs. each of the mobile phase variables.

The mix could not be completely separated at the initial temperature and pressure tried, regardless of the percentage modifier used. A modifier concentration of 13 % appeared to give the best, but inadequately resolved, results. The temperature was increased, and then decreased by 10 °C, to determine whether selectivity could be changed. It was found that lowering the temperature tended to improve peak shapes and resolution. The temperature was further lowered to 30 °C, where at least partial resolution of all the solutes was achieved.

The modifier concentration was again optimized. The resolution between the four most difficult to separate pairs of peaks was plotted as a function of modifier concentration over a narrow range of concentrations, from 8.5 to 11 % at 30 °C and 150 bar. As can be seen in Figure 6.16, the resolution of both pairs 4–5 and 5–6 improved as the modifier concentration was decreased. The resolution between pair 6–7 degraded, while the resolution of pair 3–4 stayed about the same. Only between 8.8 and 9.8 % modifier was the resolution between all the pairs greater than 1.5. The vertical dashed line in Figure 6.16 indicates that the maximum resolution between all the pairs that was obtained at 9.2 % methanol.

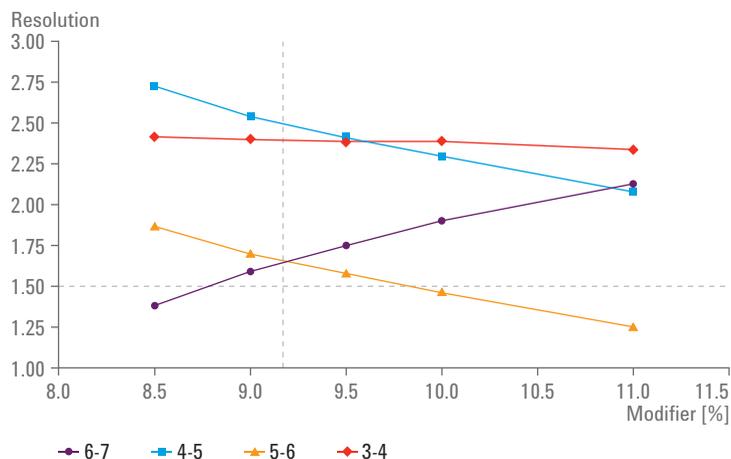


Figure 6.16 The effect of MeOH concentration on the resolution of four critical pairs in the nine-component sulfonamide mix. The highest resolution of all the pairs was achieved at 9.2 % MeOH. Resolution of some pairs increased while others decreased on over the narrow range of modifier concentration between 8.5 to 11 %.

The temperature was then varied between 25 and 40 °C, at 9.2 % methanol and 150 bar, and once again the resolution between pairs was plotted. The results are shown in Figure 6.17. The results show that peaks 6 and 7 coelute at 40 °C and that the best overall temperature was 30 °C.

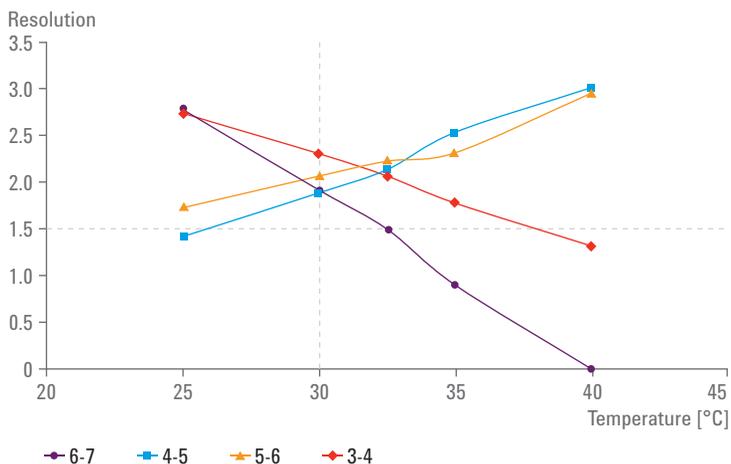


Figure 6.17 The effect of column temperature on the resolution of 4 critical pairs of the 9-component sulfonamide mix. The resolution between some pairs increased while others dramatically decreased over the 15-degree range studied. The highest overall resolution was achieved at 30 °C.

The pressure was then varied between 100 and 180 bar, and the resolution between the pairs was plotted as a function of pressure. The results, shown in Figure 6.18, indicate improving resolution at lower pressures down to 110 bar, but then degrading at lower pressures. Thus, the best overall resolution of about 2 or better for all the pairs was obtained at 110 bar. It is unusual for pressure to have such a strong impact on selectivity, but the modifier concentration is fairly low and the modifier is only modestly polar. The final optimum conditions found for maximum overall resolution were 1.7 mL/min of 9.2 % methanol at 30 °C and 110 bar.

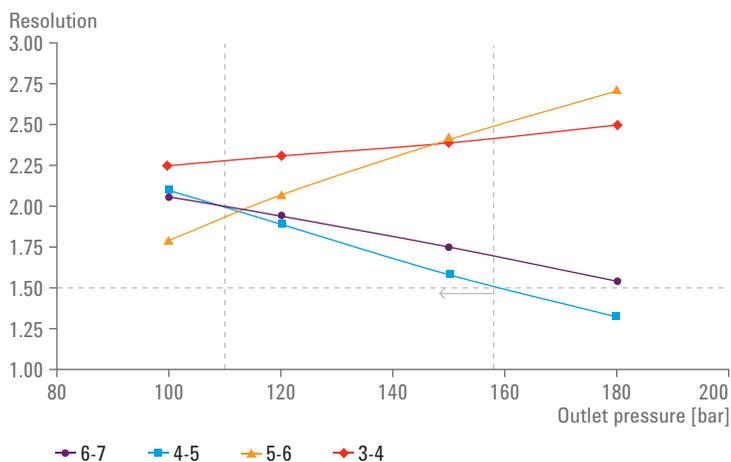


Figure 6.18 The effect of column outlet pressure on the resolution of four pairs of the nine-component sulfonamide mix. The resolution of some pairs increased, while others decreased between 100 and 180 bar. The highest overall resolution was achieved at 110 bar.

One further optimization was performed. The flow rate was also varied. Since resolution was greater than 1.5 and resolution equal to 1.5 was deemed to be the goal of the analysis, some excess resolution could be traded for speed to yield a shorter run time. The resolution of the four pairs was plotted as a function of flow rate and the results presented in Figure 6.19. Flow could be increased to 2.9 mL/min before resolution degraded to 1.5, which would decrease analysis time by 40 %. A separation optimized for speed is presented in Figure 6.20.

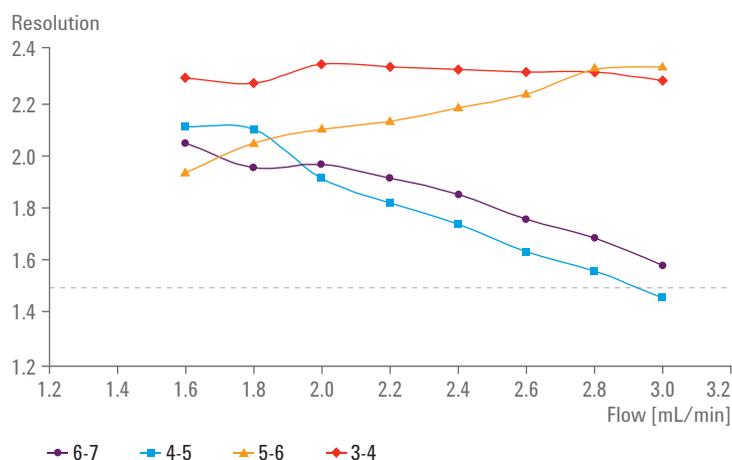


Figure 6.19 The effect of flow rate on the resolution between the four critical pairs. While the best overall separation ($R_s > 2$ for all) occurred at 1.7 mL/min, an acceptable separation ($R_s > 1.5$) was obtained at 2.9 mL/min with a run time reduced by 40 %.

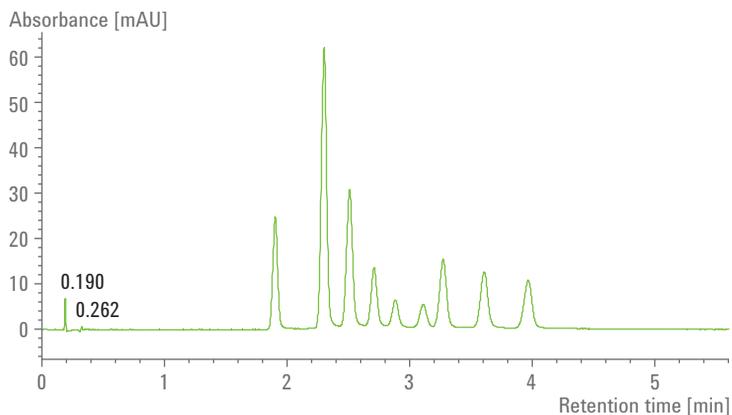


Figure 6.20 Optimized separation of nine sulfonamides with $R_s > 1.5$, for all pairs. Column: 3 by 100 mm, 1.8 μm , RX-SIL. Conditions: 2.9 mL/min of 9.2 % MeOH in CO_2 at 30 °C and 110 bar. Flow cell: 3 mm, 2 μL tapered. Injection loop: 1.25 μL . Detection: signal 260 nm with 16-nm bandwidth, reference 360 nm with 40-nm bandwidth, slit 16 nm, filter > 0.05 minutes.

6.4 Other observations

None of the above case studies used an additive. However, the need, or lack of need for an additive is usually immediately obvious. The first injection of any sample typically yields either relatively symmetrical peaks, requiring no additive, or badly distorted peaks, or no elution, requiring an additive. Once an additive is included in the mobile phase, it tends to have little impact on either retention or selectivity.

7.1 Background

The predominant use of SFC over the last 15 years has been for chiral analysis and semipreparative separations in the pharmaceutical industry, although there has been minor interest in the petrochemical, polymer, surfactant, pesticide and food industries. Most small drug-like molecules are chiral, meaning they exist in several forms that are mirror images of one another, called enantiomers. In nature, many compounds occur only as one of the forms. However, when synthesized in the laboratory, they typically occur with equal concentrations of the various forms. Mixtures with equal concentrations of enantiomers are called racemates.

It is well known that the individual enantiomers of many compounds have different physiological effects. For example, one of the enantiomers of ibuprofen is much less effective as an anti-inflammatory than the other. A pure version of the *good* enantiomer would be more effective since it might be taken at twice the dose of the racemate and, subsequently, act faster and more effectively. Alternately, the pure enantiomer could be taken as half the dose of the racemate, with the same effectiveness, but with fewer toxic side effects.

Some enantiomers can be dangerous as is the case with thalidomide, where one of the enantiomers causes birth defects. As a consequence the United States Food and Drug Administration (FDA) and other regulatory bodies have required complete testing of each individual enantiomer in any racemate.

It is possible to repatent older drugs, originally sold as racemates, but reintroduced as the pure enantiomer. This provides another 20 years of patent protection for a drug that has already survived clinical trials and therefore should be much easier and faster to commercialize. Pharmaceutical companies immediately recognized the economic incentives and began developing drugs that were pure enantiomers. Today, most new chiral drugs are sold as pure enantiomers.

Pure enantiomers are usually developed using a series of asymmetric syntheses, where each step is intended to produce only one of the enantiomers. The syntheses start with small building blocks, and progressively increase in both size and functionality, in a series of steps, until the final product is created. At each stage of synthesis, the enantiomeric purity of the intermediate product needs to be monitored

to verify that the synthesis is producing appropriately pure educt for the next synthesis step. This has led to an explosion of the use chiral chromatography at both analytical and semipreparative scales.

7.2 Enantiomeric excess determinations

If the goal is to develop a pure enantiomer, the amount of the other enantiomer needs to be quantified. Consequently, such trace analysis is common. The enantiomeric excess is used to a measure the success of an asymmetric synthesis or chiral separation. The most commonly used definition of enantiomeric excess, *ee*, is given in Equation 7.1, where *R* and *S* are the two enantiomers.

$$ee = 100 \left(\frac{R - S}{R + S} \right)$$

Equation 7.1 Calculation of enantiomeric excess.

7.3 Normal phase for chiral separations

Normal phase HPLC is more effective in separating enantiomers and other isomers compared to reversed phase, and in the past was the workhorse method in most laboratories doing chiral analyses. However, many normal phase separations use heptane mixed with other organics such as isopropanol as the mobile phase. Heptane can be a significant fire hazard, especially when used in large volumes. It is also expensive. Normal phase HPLC retention time reproducibility is highly susceptible to small amounts of water. Equilibration can take a long time. Gradients are seldom used. SFC on the other hand is at least three- to five-times faster than even reversed phase HPLC, has none of the issues with traces of water, or re-equilibration, and the CO₂ is inexpensive, as pointed out previously. Further, it is almost impossible to burn modifiers dissolved in CO₂.

A major advantage in drug discovery is that SFC can be scaled to the semipreparative level where each injection can contain as much as 100 mg of the racemic mixture, retaining the speed and pressure drop advantages at the analytical scale, as well as the low flammability, compared to normal phase HPLC. At the end of the SFC separation most of the mobile phase inherently vaporizes, leaving behind small volumes of modifier that contain the pure enantiomers, which greatly speeds up and simplifies dry-down.

The pure enantiomers obtained need first to be shown to be pure with analytical-scale chiral chromatography, and can then be used as a feed for the next stage of synthesis. At some point, each pure enantiomer must be studied in its pure form for absorption, metabolism and toxicity (ADMET) studies. Since nine out of 10 potential drugs fail, this approach is faster and less expensive than developing a series of asymmetric synthesis steps to make each pure enantiomer. Instead, a small amount of the racemic mixtures can be made and the enantiomers separated chromatographically. Thus, only the successful compounds need the time and expense to develop the series of asymmetric syntheses.

The advantages of SFC for chiral separations should be obvious. Many larger pharmaceutical companies have recognized this and many have switched most chiral separations from normal phase HPLC to SFC.

For example, in 2005, White⁸⁰ used four short 4.6 by 100 mm columns (ChiralPak AD-H, and AS-H, and ChiralCell OD-H and OJ-H) with a 2.5-minute, 15 to 55 % gradient (cycle time 6 minutes), at 5 mL/min, 120 bar and 35 °C. He preselected the modifier and additives to minimize the exposure of the chiral stationary phases to those combinations unlikely to be effective. For basic solutes, he used a basic additive in methanol, ethanol or isopropanol. However, he only ran the isopropanol when the first two failed to produce a viable separation. For acidic solutes, he substituted 0.1 % trifluoroacetic acid for the base. He compared SFC to HPLC on the hundreds of samples submitted to his analytical service laboratory for a full year to determine which technique should be scaled up for purification. At the end of the year, SFC was found to be superior in 96 % of the separations, and was subsequently adopted as the primary screening technique in all future work. As a further consequence, the largest accepted purification jobs jumped from 5 to 60 grams (faster chromatography, much faster dry-down, safer).

Of the compounds evaluated, 64 % were baseline resolved using ChiralPac AD-H, 10 % by ChiralCell OD-H, 17 % by OJ-H, and 9 % by AS-H (all from Chiral Technologies). He used a 1-minute equilibration time when the column was switched, and a 5-minute equilibration when the solvent was switched. Thus, a single solute could be screened on four columns, with one modifier-additive combination in approximately 19 minutes. The solute could be screened on all four columns, with the three different modifier-additive combinations in 54-minutes.

Maftouh⁸¹ (2005) also screened with four columns and up to six different solvents. The columns were 4.6 by 250 mm, with 10- μ m particles from Chiral Technologies, and with the phases ChiralPac AD, and AS, and

ChiralCel OD, and OJ, the same stationary phases used by White. He, like White, also preselected the mobile-phase combinations for each solute. Methanol and isopropanol were used pure, and with trifluoroacetic acid or isopropylamine. The bases, neutrals and bifunctional solutes were eluted using CO₂ with modifier and isopropylamine. The acids were eluted using CO₂ with modifier and trifluoroacetic acid.

With 500 proprietary chiral compounds the success rate was; AD 60 %, OD 31 %, OJ 8 %, and AS 2 %. The overall success rate with these four columns was 95 %. With another large set of marketed drugs, the success rate was 98 %. Conditions were 3 mL/min at 200 bar and 30 °C, with a modifier gradient. SFC was adopted as the primary analytical tool while capillary zone electrophoresis (CZE) was adopted as the backup technique.

7.4 Effect of control variables on chiral separations

Chiral separations are performed more or less the same as achiral separations. However, in most cases the solutes are present in simple mixtures, often containing only the enantiomers of interest.

7.4.1 Relative effects of each variable

Warfarin was separated on a Lux Celulose-1 column using methanol in CO₂, and the effect of modifier concentration, temperature and pressure were evaluated. The results, all shown in Figure 7.1 indicate that once again the modifier concentration is the primary means of adjusting retention. Neither temperature nor pressure had much effect on either selectivity or retention. There is a slight trend toward higher resolution at lower temperatures.

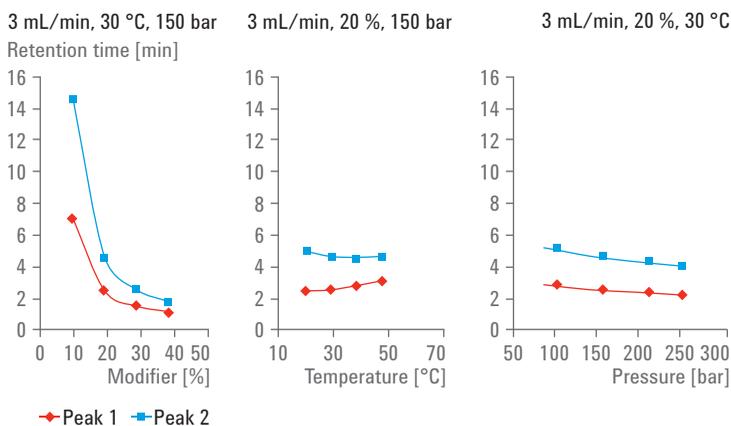


Figure 7.1 The effect of mobile phase parameters on the separation of warfarin enantiomers.

7.4.2

Conventional modifiers

As with achiral separations, methanol is the most polar solvent that is completely miscible with CO₂. As such, it is usually the first modifier used when trying to ensure that all sample components elute. Many chiral compounds, particularly starting materials and intermediates, are not highly polar and tend to elute near the void volume, even with low methanol concentrations. The obvious choice under these circumstances is to decrease modifier polarity. The usual approach is to try ethanol and then isopropanol, if the ethanol is too strong, even at 5 %. For reaching even lower polarity solutes, a relatively small concentration of isopropanol (5 to 10 %) can be added to heptane and the mixture pumped as the modifier. In this way relatively low concentrations of polar modifiers can be delivered reproducibly.

7.4.3

Bonded phases and nonconventional modifiers

In the past, many solvents could not be used as modifier, since many of the more successful older stationary phases were coated, not bonded, with cellulose or amylose derivatives. Many interesting solvents such as dichloromethane and tetrahydrofuran, could, unfortunately, wash off the stationary phase. Further, some solutes have poor solubility in the traditional solvents. There has been a significant effort over the last few years to produce bonded (immobilized) phases⁸²⁻⁸⁵, which can be used with a wider range of solvents.

Manufacturers initially attempted to duplicate the selectivity of the older phases, but this was not successful. However, bonding the phases has enabled the use of a wider range of solvents, which gives you much more control over separation and can produce significantly improved solubility. Nevertheless, these newer bonded phases have not significantly replaced the older phases in screening, but, instead, have largely been used for alternate secondary screens⁸⁴, particularly with solutes poorly soluble in the traditional solvents.

7.4.4

The need for additives

As in achiral SFC, many chiral compounds, but particularly aliphatic amines, need a highly polar additive in the mobile phase to improve peak shapes. Adderall is a commercial product used for attention deficit disorders, consisting mostly of D-amphetamine with a small amount of the L- enantiomer. These are primary aliphatic amines, as shown in Figure 7.2. The top chromatogram, acquired using 30 % methanol with no additive, shows no elution. However, the addition of 0.2 % triethylamine to the same 30 % methanol, produced a rapid, high-efficiency separation. Solute retention is highly sensitive to the concentration of modifier. It is often true in chiral SFC that doubling the modifier concentration shifts retention much more than a factor of two.

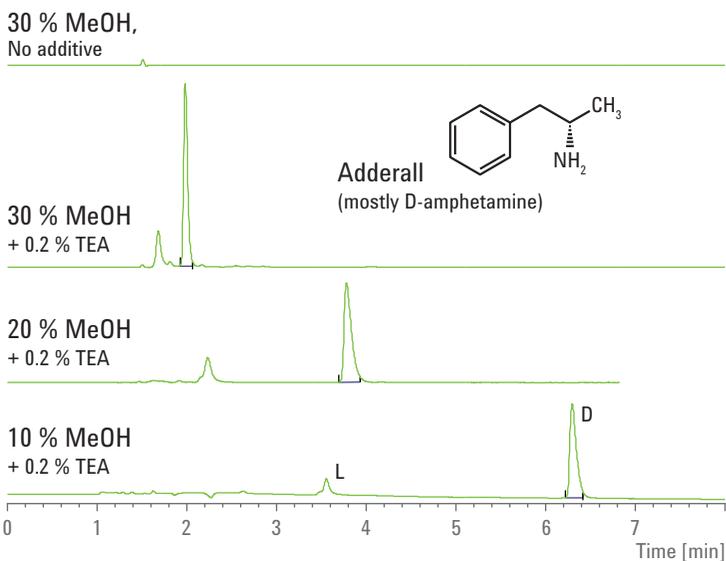
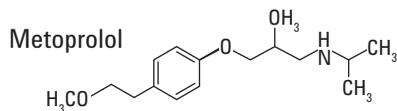


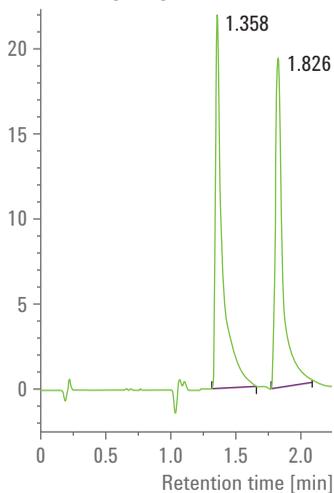
Figure 7.2 Separation of a nonracemic mixture of amphetamine, a primary aliphatic amine. Without the additive, nothing was eluted.

It was briefly mentioned in Chapter 2 “The Mobile Phase” that steric hindrance can have a significant effect on retention. Metoprolol is a secondary amine as shown in Figure 7.3. The isopropyl group tends to hinder access of the lone pair of electrons on the nitrogen to the stationary phase. The result is that the enantiomers elute with 30 % methanol and are rapidly separated, but with severe tailing. The addition of 0.1 % acetic acid to the methanol produced no change in peak shape or retention. However, the substitution of 0.1 % triethylamine resulted in symmetrical peaks and a significant shortening of the run time without loss of resolution.



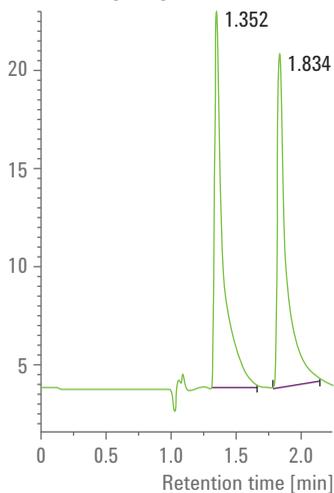
No additive

Absorbance [mAU]



0.1 % Acetic acid

Absorbance [mAU]



0.1 % TEA

Absorbance [mAU]

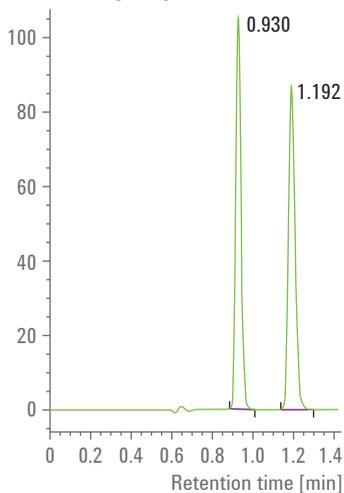


Figure 7.3 Effect of additives on the peak shape of metoprolol, a secondary amine.

Left: pure MeOH at 3 mL/min, 30 %, 30 °C, 120 bar; Center: same conditions but with 0.1 % glacial acetic acid added to the MeOH. Right: same conditions but with 0.1 % triethylamine added to the MeOH. Column: 4.6 by 250 mm, 5 μ m Regiscell.

The less hindered tertiary aliphatic amine, mirazapine, did not elute with 40 % methanol without additive, see Figure 7.4. However, the addition of triethylamine resulted in the elution of sharp peaks, with little retention (k about equal to 1), or resolution. Decreasing the modifier concentration resulted in significant increases in both retention and resolution.

Remeron
(mirtazapine)

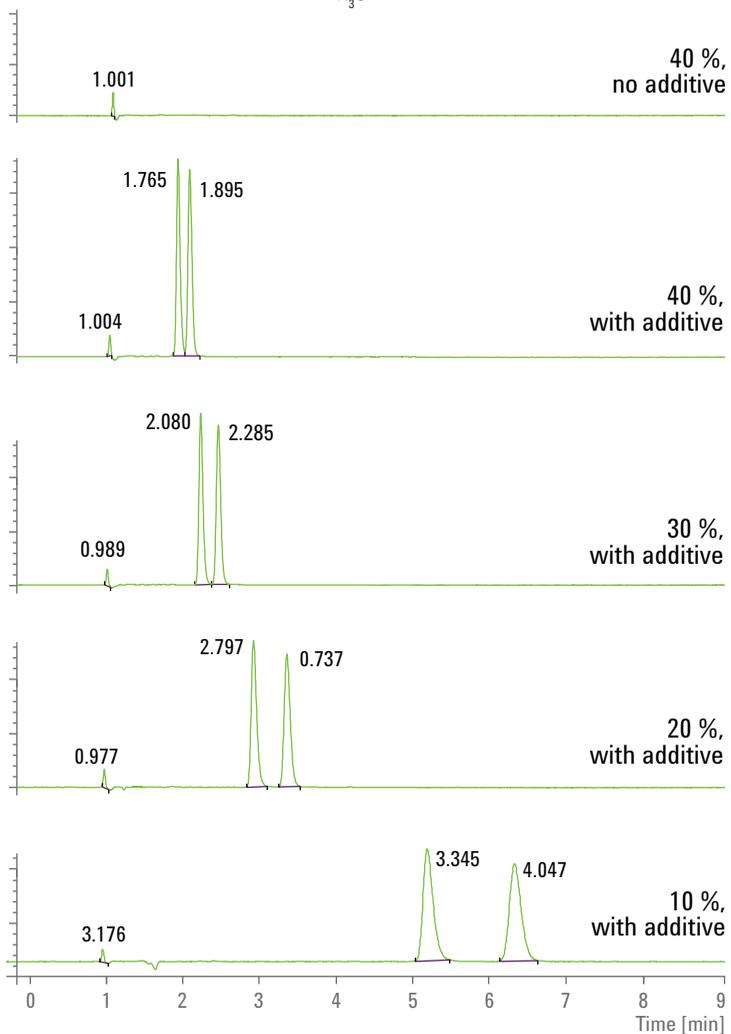
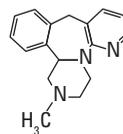


Figure 7.4 Separations of the tertiary amine remron (mirtazapine) as a function of modifier concentration and the presence or absence of additive. Without additive nothing eluted. 3 mL/min of various percentages of MeOH + 0.1 % TEA, 40 °C, 150 bar. 4.6 by 250 mm, 5 μ m Chirapak AD-H.

It is interesting to note that the need for additives in these three examples is very different from the behavior of the compounds used to generate Figure 6.11, where the nitrogens were in rings but the lone pairs of electrons were conjugated with either a carbon-carbon, or a carbon-oxygen double bond. In those cases, no additive was required.

It should be remembered that additives strongly adsorb onto stationary phases and may be difficult or even impossible to wash off. For additives that can be removed, care should be taken not to switch directly from an acid to a base or vice versa, since this may generate nonreproducible results. There are also claims of irreversible changes to some chiral stationary phases after an additive was used. Many analysts keep two sets of columns, one for acids and the other for bases.

7.4.5

Temperature

Enantiomers are chemically identical and differ from each other only in shape. Lower temperatures tend to improve chiral recognition by decreasing kinetic energy. Gasparinni⁸⁶ performed chiral separations as low as $-50\text{ }^{\circ}\text{C}$ with increasing enantiomeric resolution, but at such low temperatures the mobile phase tends to become highly viscous. It is generally agreed that low temperatures do not increase throughput significantly. Most standard ovens have a lower temperature limit of 20 to 30 $^{\circ}\text{C}$. Further, at substantially lower temperatures condensation of water from the lab air can be a problem. Consequently, most chiral separations in SFC have been performed between 30 and 40 $^{\circ}\text{C}$.

7.5

Developing a chiral method

7.5.1

Choosing a stationary phase

It is still true that you cannot predict which stationary phases will separate a specific set of enantiomers. Consequently, selection of an appropriate chiral stationary phase is reduced to trial and error. Screening systems have been developed to try to automatically find the best combination of mobile and stationary phases. The most common approach is to use automated column and solvent-selection valves, along with a software wizard that allows you to choose which columns and mobile phases to try for each specific compound.

Although there are many chiral stationary phases available, both White⁸⁰ and Maftouh⁸¹ had over a 95 % success rate with only four of the older coated stationary phases. Many other workers have had similar success. This suggests that any initial screen can start with a relatively small number of stationary phases, to minimize wasted time and materials. It is fairly common to have four to six different chiral stationary phases on a single valve, or two matched valves that are switched simultaneously. The chiral stationary phases are usually tried serially, using the same mobile phase.

Others have used valves with more ports, or several valves in series. For example, Welch⁸⁷ described an SFC system using two 14-port valves. Five columns and a bypass were mounted on each valve, which allowed evaluation of 10 individual columns *or* 25 combinations of two columns in series. Others have developed similar noncommercial systems with up to 19 different columns.

Faster screening methods continue to be developed with shorter columns and smaller particles. Hamman⁸⁸ recently (2011) compared six different 4.6 by 50 mm columns packed with 3- μ m particles, using a 1.5-minute gradient from 10 to 55 % modifier, with a 1-minute hold at 55 %. The columns were AD, Cellulose-1, OJ, IC, AS, and Cellulose-4. Three different modifiers were available: methanol, ethanol, and isopropanol. If the solutes were a base, 0.1 % diethylamine, or triethylamine was added to the modifier. Again, the authors preselected the modifier and additive combinations. The authors state a six-column screen took 20 minutes (six columns, single modifier).

There are also several parallel systems where flow from a large pump is split into up to eight streams, each directed through a different column. Each column sees the same mobile phase at the same time. The injection is also split. There are specialty detectors with up to eight multiplexed flow cells. Alternately, individual detectors for each channel have also been used. Thus, each solute is evaluated on up to eight different columns in a single run time. One problem with such systems is the lack of versatility.

7.5.2

Choosing a mobile phase

In the choice of mobile phase there are two questions: First and most importantly, is an additive needed? Second, how polar does the modifier need to be?

Regarding additives, it is advisable to group solutes into acids, neutrals, bases, and bifunctional groups, such as White⁸⁰, Maftouh⁸¹, Hamman⁸⁸ and many others have done. If an additive is required on one chiral stationary phase, the additive will likely be required on all similar chiral stationary phases, regardless of how polar or nonpolar the solute is. It makes little sense to automatically run pure modifiers, plus both acidic and basic additives on every solute as part of an initial screen, particularly when the pure modifier is run first. Use of a pure modifier may not elute all the solutes, which could then build up on the column, potentially changing selectivity.

Repeatedly switching back and forth between acidic and basic additives for each solute is likely to cause damage to the columns, and potentially yield nonreproducible results, particularly when careful change-over procedures are not followed. The most conservative approach is to use a strong base as additive for basic solutes, and a strong acid for acidic solutes, with careful preconditioning during change-over from one additive to the other. Alternately, you can use two different sets of columns, one for acidic additives, and one for basic additives.

7.5.3 Other variations

Medvedovici⁸⁹ screened 44 chiral compounds with a broad range of functional groups using six different chiral stationary phases, of three different types, and a 5 to 30 % methanol gradient containing either trifluoroacetic acid or triethylamine. Chiralcel OD resolved 66 % of the pairs. Chiralpak AD resolved 70 % of the compounds. A Chirobiotic V column resolved 48 %, while Chirobiotic T resolved 50 %. The brush-type phases did not perform as well, with the Chirex 3022 resolving 34 %, but the Chirex 3005 resolving only 20 %.

Welch⁹⁰ created a diverse library of 48 racemates, which he stored in 96-well microtiter plates specifically to evaluate new chiral stationary phases. A universal method, of CO₂ with 4 to 40 % (methanol and isobutylamine) was used. Only a basic additive was used. ChiralPak AD-H baseline resolved 60.4% of the solutes, while OD resolved 30%, almost identical to Maftou's findings⁸¹, with different solutes.

In an alternate approach, Zhao⁹¹ in 2003 pooled up to 12 racemic mixtures and used SFC-MS to identify the peaks, based on mass. Acids, bases and neutral compounds were segregated. The Acids were separated using methanol and trifluoroacetic acid. The bases and neutrals were separated using methanol and isopropylamine. Four different columns (Chiralpak AD, AS, and Chiralcell OD, and OJ) were sequentially screened. The mixed sample was separated using eight different concentrations of modifier over 20 minutes, starting at 40 %, then decreasing by 5 % per run. Flow was 2.5 mL/min, temperature was 35 °C, with 110-bar outlet pressure. Success was reported in the order AD—AS—OJ—OD.

Also in 2003, Garzotti⁹² used electrospray MS in conjunction with SFC to separate a broad range of racemates and also differentiated the peaks based on mass. He used Chiralpak-AD, and Chiralcell-OD, and Chiralcell-OJ at 35 °C, 180 bar and 2.5 mL/min. He claimed AD resolved 80 %, OD resolved 45 % and OJ resolved 50 % of the racemates studied. He mostly used ethanol with isopropylamine as additive. He injected mixed standards of up to four racemic mixtures. He stated there was a

significant increase in stereoselectivity with decreasing temperature, but pressure had almost no effect on resolution.

The most effective chiral stationary phases described above are nonbonded, which limits the range of modifiers that can be used without washing the phase off the column. New bonded phases attempting to reproduce the selectivity of the nonbonded phases have resulted in variable results. There is no one-to-one replacement. However, these newer bonded phases enable the use of most organic solvents as modifier and sample solvent and are becoming popular.

7.5.4

A method development system

The Agilent method development system uses two or three thermostatted column compartments with four or six independent heat exchangers, capable of controlling temperatures between 10 degrees below ambient and 100 °C. Two of the column compartments contain an 8-position/9-port switching valve for column selection. Up to eight columns can be fitted. Each column inlet is connected to one of the ports through one of the heat exchangers. All the column inlets are connected to the same valve, while all the column outlets are connected to the other valve. The valves are switched in unison to place successive columns in the flow path.

Several low dispersion heat exchangers can be mounted on each of the standard heat exchangers, significantly decreasing the lengths of tubing required to connect the columns to the valves. For rapid screening up to four low dispersion heat exchangers, and four 100-mm long columns, can be mounted in a single column compartment. Longer columns can also be mounted in each column compartment. Combinations of both short and long columns can be installed. The shorter columns can be used for rapid screening to identify the best stationary phase for a separation, and then the appropriate longer column can be used for higher resolution work.

Recently, an alternative approach has been offered by Agilent that uses a single column compartment equipped with a valve that enables five columns and a bypass to be connected in parallel. A single valve with small ports is used to connect both column inlets and outlets. This significantly shortens the lengths of tubing required to connect the columns to the valve, which decreases extracolumn band broadening.

Solvent selection can also be automated with a 12-position/13-port valve, meaning up to 12 different modifiers could be tried. Thus, three modifiers such as methanol, ethanol and isopropanol could each be tried pure, with a basic additive, or with an acidic additive. Both column and solvent selection valves can be controlled through software, which facilitates

rapid development of sequences. Setup is simple. Column identities are listed in one table. Modifier identity is listed in another.

It is often true that specific, well-established methods are used routinely, but not on a continuous basis. A method development system can be used in support of a group of medicinal chemists each primarily performing asymmetric syntheses. Instead of having a large number of intermittently used machines, or continuously changing columns for each different method, the system can be configured with the most common stationary and mobile phases. Each user can then submit a sample and the name of their method (column and modifier combination) for automated analysis.

8.1 Stages of Validation

In the past, SFC was seldom used in routine QA/QC applications because the dynamic range of UV detectors was inadequate to monitor both major, minor and trace components in the same run, and since UV noise was on the order of 0.5 mAU with a filter setting of greater than 0.1 minutes. This was up to 50-times worse than HPLC. This meant that a minor component, representing 0.1 % of a major component having an absorbance less than or equal to 1000 mAU, could not be quantified with a signal-to-noise ratio of greater than 10. Such quantification requires a noise level less than 0.1 mAU. In the recent past, such problems have been largely solved by significantly decreasing UV detector noise by as much as a factor of 50, as described in detail in Chapter 9 "Instrumental Considerations". Routine analysis accounts for up to 10-times more users compared to method developers. This is potentially a large and new application area for SFC.

In order to perform routine analysis, both the equipment and the method must be shown to be robust. That is, they must be shown to adequately perform the tasks demanded of them, over multiple days, months or years, from lab to lab, instrument to instrument, and so on. This includes dynamic range, sensitivity, linearity, accuracy, precision and repeatability.

There are multiple texts devoted exclusively to validation, which means any detailed discussion here, in a short chapter, can only be inadequate. However, an overview is provided, with the suggestion that you should consult such texts.

8.1.1 Design qualification

Design qualification (DQ) is about whether the manufacturer understood the needs of the customer and designed the equipment to meet those needs. This is largely about comparing specification sheets describing the performance of the hardware, written by the manufacturer, to the customers' needs, as perceived by the customer.

8.1.2 Installation qualification

Installation qualification (IQ) is usually performed by the instrument manufacturer, where, after they perform the installation, they demonstrate the hardware at least initially meets the design specifications as stated by the manufacturer. The end user and the manufacturer need to agree, *a priori*, to how to describe *success*. Flow, pressure, temperature, ranges, precision, accuracy, detector characteristics, and so on, were previously

specified in the DQ process, and the means to measure these parameters also needed to have been agreed to at the DQ level. Remember that SFC differs from HPLC in that the SFC mobile phase remains highly compressible. Terms such as *flow rate* and *% modifier* need to be carefully defined. Preferably, a chromatographic test is used to confirm performance based on retention times and resolution between the components of a standard sample on a standard column, under a test protocol. If properly designed, such a test confirms flow accuracy, composition accuracy, temperature accuracy, pressure control accuracy, and so on.

8.1.3 Operational qualification

Operational qualification (OQ) is closely associated with routine maintenance and is often repeated regularly. Here, the primary question is: *is the instrument operating within the agreed upon specifications, at this time, and for this method?* This is both an initial and an ongoing question. Once again, a standard test sample along with a standard test column (*only used for this purpose*), can show if the system is performing properly.

Qualification teams sometimes wish to use various meters to directly measure temperature, pressure, flow, composition, or other parameters. Such meters need to be calibrated at least annually using traceable standards. However, it is often true that the accuracy of available meters is worse than the accuracy and repeatability achievable by using a standard sample and standard column and viewing the chromatographic results.

8.1.4 Performance qualification

Performance qualification (PQ), or performance validation (PV) (which usually means the same thing) is more about the quality of the current instrumental method and usage of the instrument, although there is some argument whether this actually constitutes several substeps.

To validate a method, the user needs to demonstrate that the accuracy, precision, resolution, linearity range and robustness of the method can repeatedly produce the desired result.

There are internationally agreed standards as to what an analytical method must achieve in order for the results to be recognized as valid. In the following exercise, a method is developed and validated for the quantification of sorbate and benzoate (widely used food preservatives) and caffeine in various beverages and a few viscous foods, to at least vaguely address the needed steps.

Later, a trace method for the chiral separation of flurbiprofen is demonstrated.

8.2

Method development for quantification of sorbate, benzoate, and caffeine in beverages and foods

8.2.1

A statement of the problem

In order to demonstrate a typical routine quantification of major and minor components, several preservatives and plus caffeine were determined in a number of beverages and foods. Benzoic and sorbic acids, and their sodium, potassium and calcium salts are used as preservatives in acidic foods. They are widely used⁹³⁻⁹⁵ in soft drinks, fruit juices, other beverages, soy sauce, catsup, salad dressing and many other processed foods. They are banned for use in fresh meats but are used in some sausages. They inhibit the growth of molds and yeasts, and are also effective against a wide range of bacteria, which explains their widespread use and major health benefits.

Both sorbate and benzoate are regarded as safe. Sorbate has an additional advantage since it has no taste, and has shown less toxic side effects than benzoate, since it is present in human metabolism. Nevertheless, some adverse reactions have been noted, which have resulted in limitations on the concentrations that can be used in foods. In the USA, the limit for benzoic acid is less than 0.1 %, expressed on food labels as *less than 1/10 of 1 % sodium benzoate*. There is no legal limit on sorbate in the USA, but one international agreement sets a maximum level of 0.3 %. European Union Directive 95/2/CE is much more restrictive, having established the limit for benzoic acid at 150 mg/L (0.015 %), and sorbic acid at 300 mg/L (0.03 %), if used separately, and 150 and 250 mg/L (0.015 and 0.025 %) if used together. These limits are expressed as benzoic and sorbic acids (not the salts).

8.2.2

Some background (an aside)

As a result of public concern and the near ubiquitous use of these additives, a group of countries, including the USA, UK, and China have established a level of maximum annual exposure. This results in huge numbers of analyses per year. As part of this program, the actual levels of these additives in various foods are routinely measured, so that total annual dietary intake can be estimated. Several other countries, such as Brazil⁹⁴, Portugal⁹⁶, and New Zealand⁹⁷, have performed similar studies.

Several techniques have been used, including gas chromatography^{98,99} with derivatization, various spectroscopic methods¹⁰⁰, and capillary electrophoresis¹⁰¹. However, HPLC with ion pairing is most widely used^{102,103}, and appears to be the method of choice. Nevertheless, peak shapes and resolution have tended to be poor, with 10 to 20-minute analysis times¹⁰⁴⁻¹⁰⁷. Acidified acetonitrile is still used with a C18 column. New HPLC methods continue to be developed.

Surprisingly, there is no reference in the literature to the use of SFC for the analysis of preservatives such as benzoate or sorbate in foods and beverages, although benzoic acid was often used as a test probe in the early days of SFC, and several papers have appeared related to the separation of hydroxyacids¹⁰⁸ and polycarboxylic aromatic acids¹⁰⁹. SFC has seldom been used for the analysis of aqueous samples, which may explain this lack of references. However, many samples can be diluted with methanol, filtered or centrifuged, or both, and then injected without further pretreatment.

Benzoic acid has a UV wavelength maximum near 227 nm with a small lobe near 280 nm. Sorbic acid has a UV maximum at 255 nm, while caffeine has a maximum at 280 nm. Most studies have used a single wavelength near 235 or 280 nm when all three are present. However, for optimum detection it is relatively straightforward to measure each compound present using its optimum wavelength.

Other food additives are sometimes present, which can complicate the analysis^{110–113}. The most common are caffeine, saccharin, and one or more of the parabenes. They could be regarded as interferences in the benzoate-sorbate acid assay, but are usually included in the measurements.

8.2.3 Purpose

The purpose of the present work is to develop a method and demonstrate many of the steps required to validate it for the quantification of benzoate, sorbate and caffeine in several aqueous-based beverages and foods using SFC.

8.2.4 Development of the chromatographic method

Since there were no reports in the literature on the separation of benzoic and sorbic acid, as well as caffeine, it was necessary to develop a method. Preliminary work showed that the analytes could be eluted using relatively low concentrations of methanol as modifier, although benzoic acid tailed severely without an acid additive. Both trifluoroacetic acid and acetic acid were shown to improve the peak shapes of both benzoic and sorbic acids. Acetic acid was chosen for further work, since it is less expensive, more common, greener, and safer to work with. The column temperature was arbitrarily set at 50 °C. The outlet pressure was chosen as 150 bar to avoid any noise issues associated with significantly lower pressures. The autosampler was set to overload the 5- μ L loop with 15 μ L of standard or sample.

Initial experiments using a ZORBAX RX-SIL column showed that resolution between sorbate and benzoate was only about 1.0 at 7.5 % (methanol and 0.3 % acetic acid), with little retention, while at the same time, there was excessive time between benzoate and caffeine.

A *Premier* column, specifically designed for separation of acids in SFC, produced exceptional selectivity, with high resolution as shown in the top chromatogram in Figure 8.1. However, caffeine was almost unretained, and the separation of the preservatives required 40 % organic modifier. Methods where components are essentially in the solvent front need to be avoided. A diol column produced a significantly improved separation, eluting all three analytes in less than 2 minutes with resolution of greater than 4, and 2.64, between the two pairs, while requiring only 8.5 % (methanol and acetic acid), as shown in the bottom chromatogram in Figure 8.1.

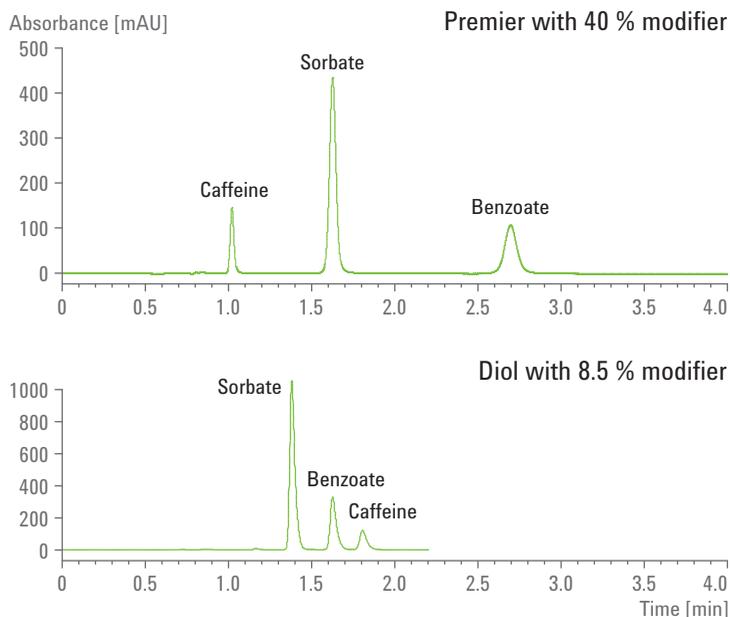


Figure 8.1 Separation of benzoate, caffeine and sorbate at high concentrations.

Top: Separation using a *Premier* 4.6 by 250 mm, 5- μ m SFC column at 3.5 mL/min of 40 % MeOH containing 0.3 % acetic acid in CO₂, at 50 °C with 150-bar outlet pressure. Bottom: Same separation on a 4.6 by 250 mm, 5- μ m diol column with the same conditions except at 8.5 % modifier. 5 μ L injected, 235 nm, 16 nm, slit filter greater than 0.03 minutes. Resolution was greater than 4 and 2.64 on a diol column.

The retention order on the Premier column was different from the retention order on the diol column. Such large differences in selectivity can be used to help ensure that no interference peaks are coeluting with any of the solutes since the methods are orthogonal.

Composition, temperature and pressure were changed to slightly higher and lower values, which demonstrated robustness and reproducibility in retention times and resolution. Since the resolution with 8.5 % modifier was greater than 1.5, the method was deemed acceptable for major and minor component analysis.

8.3 Calibration

8.3.1 Designing the calibration curves to meet the analytical requirements

In order to validate this method, a calibration curve needs to be generated. Details such as the number of concentrations of standards, the number of injections per standard, the precision of the analysis with each of the standards, the linearity of the calibration curve and the linear range all need to be evaluated and shown to be adequate.

Calibration curves should include standards that bracket the expected results by at least $\pm 30\%$. There should be several standards both higher and lower in concentration compared to the expected result. With these boundary conditions, it is feasible to try to make appropriate calibration curves for the chosen analytes in this example.

The legal limit on benzoate in the USA is less than 1/10 of 1 %, or less than 0.1 %. Here, the aqueous samples were diluted 3:1 to avoid any miscibility issues between the water and the mobile phase, so the upper limit found in the samples should be less than 0.25 mg/mL. The highest concentration used to generate the calibration curve was 0.5 mg/mL or twice the expected maximum. Standards were also prepared at 0.35, 0.25, 0.15, 0.075, 0.05 and 0.025 mg/mL with multiple standards both above and below the expected value.

There is no legal limit on sorbate but one international agreement indicates the maximum permitted should be less than 0.3 % (3 mg/mL). As with benzoate, the samples were diluted 3:1 so the upper limit of the calibration curve ought to be 1.3 to 1.5-times 0.75 mg/mL.

There is a requirement that the amount of caffeine added to many beverages must be listed on the label. The labels generally list the caffeine content as the total weight of caffeine in the container. For the samples tested that listed their caffeine content, the values ranged between 0.0529 to 0.336 mg/mL, making 0.5 mg/mL adequate for use as the upper limit of the calibration curve, but also required an extended calibration curve to lower values since beverages contain a wide range of concentrations.

Each of the beverages and foods had 1, 2 or 3 of the analytes present, meaning one or more were often absent. Thus, it was interesting but not necessary to estimate the limit of detection and the limit of quantification of each of the analytes in each sample. Most of the samples contained either sorbate or benzoate but there is little interest in sorbate or benzoate concentrations significantly below the legal or recommended limits. However, since there were several beverages with reportedly a wide range of caffeine concentrations, the calibration curves were extended to cover the lowest concentration expected.

8.3.2

Creating the calibration curves

A total of seven standards and a blank, bracketing the concentrations found, were used. The standards contained all three analytes at equal concentrations. Each analyte was monitored at its wavelength of maximum absorbance. The standards covered a dynamic range of 20 between 0.025 and 0.500 mg/mL.

8.3.2.1

Precision

The precision of analysis of each standard was determined by making six injections at each concentration, and the RSDs for both retention time and area counts were determined. The RSDs for retention time for all three analytes averaged 0.048 % with a maximum value of 0.08 %, as shown in Table 8.1.

Concentration	Sorbate			Benzoate			Caffeine		
	Mean	SD	RSD [%]	Mean	SD	RSD [%]	Mean	SD	RSD [%]
0.5 mg/mL									
RT	1.3768	0.000373	0.03	1.6215	0.000548	0.03	1.7998	0.001169	0.06
Area	3337.0	4.3191	0.13	855.2	0.7183	0.08	707.3	0.9208	0.13
0.35 mg/mL									
RT	1.3767	0.00047	0.03	1.6222	0.00040	0.03	1.7982	0.00098	0.05
Area	2408.2	5.5694	0.23	605.27	2.0519	0.34	497.9	1.4125	0.28
0.25 mg/mL									
RT	1.3763	0.00047	0.03	1.623	0.00081	0.05	1.7955	0.00104	0.08
Area	1757.5	1.5302	0.09	439.23	0.5465	0.12	360.03	0.5316	0.15
0.15 mg/mL									
RT	1.376	0.00057	0.04	1.6234	0.00051	0.03	1.794	0.00063	0.04
Area	1051.13	0.69186	0.07	261.9	0.54406	0.21	213.73	0.41312	0.19
0.075 mg/mL									
RT	1.3752	0.00068	0.05	1.624	0.00063	0.04	1.7923	0.01033	0.06
Area	513.23	0.5750	0.11	128.12	0.2562	0.20	104.4	0.2280	0.22
0.05 mg/mL									
RT	1.3737	0.00094	0.07	1.6242	0.00075	0.05	1.7895	0.01049	0.06
Area	322.77	0.1505	0.05	80.38	0.3600	0.45	65.33	1.1032	0.16
0.025 mg/mL									
RT	1.3738	0.00098	0.07	1.6242	0.00098	0.06	1.7888	0.00098	0.05
Area	174.95	0.2429	0.14	43.75	308.22	0.07	35.67	0.1663	0.46
Blank	All 000								

Table 8.1 The mean values, standard deviations, and RSDs for retention times and area counts, with six injections of each, for the sorbate, benzoate and caffeine standards at concentrations between 0.025 and 0.5 mg/mL used to generate the calibration curves. Due to the short run times, the calibration curve, with seven standards and a blank, consisting of 48 runs could be generated in approximately 2.4 hours with an isocratic run time of 3 minutes.

The RSDs for area were all found to be under 1 % as also shown in Table 8.1. As expected the RSDs for the higher concentration standards were relatively low, typically less than 0.25 %. At the bottom end of the concentration range, the RSDs became larger but still under 1 %. Both sets of measurements were found to be acceptable for quantifying the analytes over the ranges expected.

8.3.2.2 Linearity

The mean of the area counts at each concentration was plotted for each analyte, and the data was also subjected to linear regression. The plots of area against concentration showed straight lines with different slopes as shown in Figure 8.2. The regression showed linear correlation coefficients of greater than 0.999 in all three instances, as shown in Table 8.2. Linear correlations greater than 0.990 are deemed acceptable. Intercepts were relatively small.

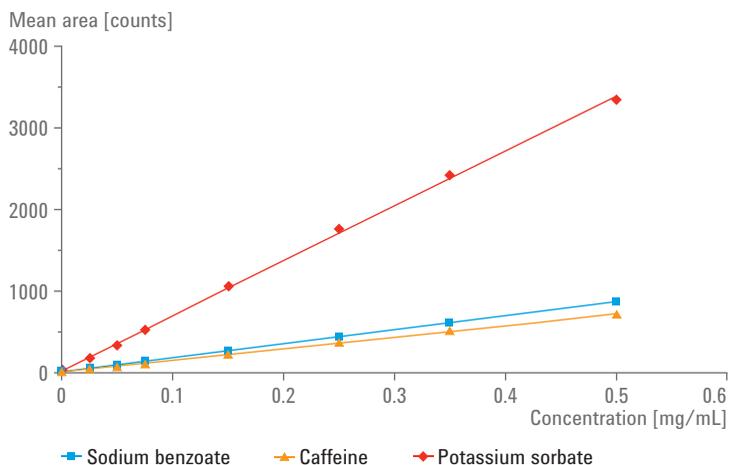


Figure 8.2 Calibration curves for potassium sorbate, sodium benzoate, and caffeine collected at 255, 235 and 280 nm respectively.

Diol, 5 μm , 4.6 by 250 mm			
	Sorbate	Benzoate	Caffeine
r^2	0.999574	0.999876	0.999925
Slope	6750.76	1721.29	1422.41
Intercept	14.23	0.51	-0.88

Table 8.2 The linear correlation coefficients, slopes and intercepts for the three calibration curves for benzoate, caffeine and sorbate used in this analysis. The linearity was exceptional. The intercepts were acceptable. The data for each analyte was collected at different wavelengths: sorbate at 255 nm, benzoate at 235 nm, and caffeine at 280 nm.

8.3.3 Samples

8.3.3.1 Beverages

15 commercially bottled beverages were randomly purchased as local markets for analysis. Approximately 20 mL of each sample were placed in a 40-mL scintillation vial. Carbonated beverages were vigorously shaken not stirred with the cap tightly closed to partially degas. The caps were loosened slightly to allow the gas to escape. The caps were retightened, and shaken again, for a total of three times. All the samples were then sonicated for 12 minutes.

A 1-mL aliquot of each sample was transferred into a 25-mL plastic beaker, using an Eppendorf pipette, then 3 mL of methanol were added. The beakers were covered with a small watch glass and sonicated for 12 minutes. Approximately 1.5 mL of each sample was then transferred into an amber-colored 2-mL autosampler vial and closed with a PTFE-lined crimp cap for analysis.

8.3.3.2 Foods

Many of the food samples were viscous (catsup for example) or contained significant solids (Italian salad dressing). For these samples, 1 g was weighed into a 40-mL scintillation vial and 10 mL of acidified methanol was added. The samples were shaken thoroughly for 30 seconds and sonicated for 12 minutes. The solutions were allowed to settle and the supernatant liquid was transferred to 15-mL centrifuge tubes with closures. The tubes were centrifuged for 10 minutes at 3200 rpm. Approximately 1.5 mL of the resulting supernatant liquid was transferred to amber vials, in the same manner as the other samples. All the standards and samples were analyzed within a few hours of preparation.

8.3.4 Analysis of samples

Similar to the standards, six injections of each sample were also made and the RSDs determined. The standards and samples were run as part of a single sequence. Each of the samples was typically present as one of several salts. Accurate quantitative analysis depended on which salt was listed on the label with an appropriate correction.

8.3.4.1 Beverages

A typical chromatogram representing a tea is presented in Figure 8.3. There was little caffeine present. Noise was of the order of 0.05 mAU with a filter setting of greater than 0.03 minutes. The concentration of caffeine was found to be 0.035 mg/mL. Thus, the limit of quantification for caffeine using this method (with signal-to-noise greater than 10) would be about 0.0105 mg/mL. The limit of detection with signal-to-noise greater than 3, would be 0.0032 mg/mL.

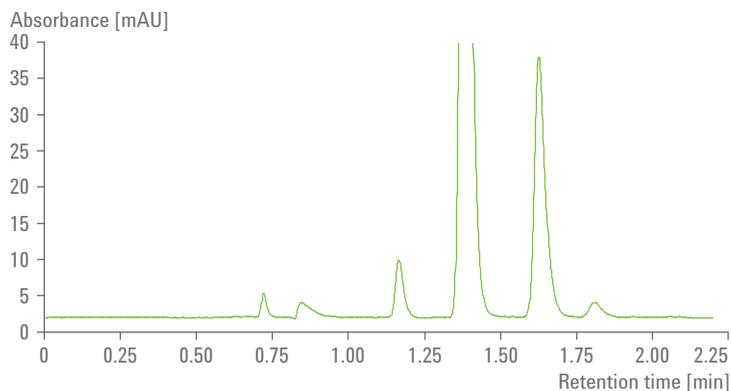


Figure 8.3 Chromatogram of a tea containing both sorbate and benzoate with a low concentration of caffeine. The caffeine was present at about 3.33-times the LOQ (S/N greater than 10) at 0.0105 mg/mL.

The results for the beverages suggest there might be greater regulatory oversight compared to some of the prepared foods. Of the 15 beverages analyzed, all were properly labeled, in terms of which of the preservatives was present, or not, as shown in Table 8.3. Further, none of the samples exceeded any legal or agreed-to limit regarding benzoate or sorbate. However, some had RSDs as high as 2.99 % probably due to matrix effects. While such RSDs are relatively high, in terms of the relatively high concentrations of the preservatives, it is probably acceptable. As usual, samples with higher concentrations had lower RSDs with respect to area count.

	Sorbate			Benzoate			Caffeine		
	Concentration [mg/mL]	RSD	On label	Concentration [mg/mL]	RSD	On label	Concentration [mg/mL]	RSD	On label
Cola	NF	NF		NF	NF		0.094	2.99	Yes
Cola diet	NF	NF		0.193	1.15	Yes	0.131	0.95	Yes
Cola zero	NF	NF		0.191	1.58	Yes	0.098	1.18	Yes
No caffeine	NF	NF		0.044	0.52	Yes	NF	NF	
Decaffeinated zero	NF	NF		0.056	0.59	Yes	NF	NF	
Soda #1	NF	NF		0.387	0.21	Yes	0.153	0.20	Yes
Soda #1 diet	NF	NF		0.456	0.13	Yes	0.161	0.11	Yes
Tonic	0.114	0.55	Yes	NF	NF		NF	NF	
Tonic diet	NF	NF		0.384	0.09	Yes	NF	NF	
Energy diet	NF	NF		NF	NF		0.324	0.20	Yes
Punch	0.355	0.18	Yes	NF	NF		NF	NF	
Apple	NF	NF		NF	NF		NF	NF	
Tea diet	0.267	0.14	Yes	NF	NF		0.049	0.45	Yes
Tea #1	0.303	0.08	Yes	0.212	0.48	Yes	0.035	0.64	
Tea #2	0.130	0.32	Yes	0.247	0.23	Yes	0.024	1.89	Yes

Table 8.3 Sorbate, benzoate and caffeine content in various beverages. The preservative content of all the beverages was found to be consistent with their labels. The US limit for benzoate is 1.0 mg/mL.

With caffeine, there was a large range of concentrations present. For these samples, the concentration found was compared to the label claim, and the results are presented in Table 8.4. The concentration of caffeine in all the samples was fairly close to the label claims, with a range of values from 93.2 % ($\Delta = -6.8\%$) of the label value to 106.8 % ($\Delta = +6.8\%$) of the label value.

Product	Label specification	Amount found [mg]	Amount found [%]
Cola	57 mg/20 oz	53.1 ±1.59	93.2 ±3.0
Diet cola	76 mg/20 oz	74.0 ±0.70	97.4 ±0.09
Cola zero	57 mg/20 oz	55.7 ±0.66	97.7 ±0.12
Soda	91 mg/20 oz	86.8 ±1.74	95.4 ±2.0
Diet soda	91 mg/20 oz	91.2 ±0.10	100.2 ±0.11
Diet energy	80 mg/8.4 oz	77.1 ±1.54	96.4 ±2.0
Diet tea	12 mg/8 oz	11.2 ±0.05	93.3 ±0.45
Tea #1	Not given	8.02 ±0.05	Not applicable
Tea #2	5 mg/8 oz	5.34 ±0.10	106.80 ±1.9

Table 8.4 Caffeine content found in several the beverages analyzed, compared to concentrations on the label. The average found was 97.6 % of the given specification.

8.3.4.2 Foods

Several semiliquid foods such salad dressings and condiments were also evaluated for their content of the preservatives. Only one contained caffeine and it was most likely not added intentionally, since it was chocolate syrup and chocolate naturally contains caffeine.

Unlike the beverages, many of these condiments were grossly mislabeled in terms of which preservative were present, as shown in Table 8.5. Perhaps the most egregious miss-labeling occurred with a soy sauce, duck sauce and a Chinese mustard from a Chinese take-out restaurant. All were labeled as containing less than 1/10 of 1 % sodium benzoate, which to a point was true, since they all contained no detectable trace of benzoate, but all were found to have large concentrations of sorbate, which was not listed on their labels.

	Sorbate			Benzoate			Caffeine		
	Concentration [mg/g]	RSD	On label	Concentration [mg/g]	RSD	On label	Concentration [mg/g]	RSD	On label
Lime juice	NF	NF	No	1.251	0.29	Yes	NF	NF	
Chocolate syrup	0.941	0.70	Yes	NF	NF	No	0.246	18.85	Natural
Italian	0.665	0.72	No	0.479	1.11	Yes	NF	NF	
Balsamic	0.841	0.92	No	0.910	1.46	Yes	NF	NF	
Ranch	1.411	0.89	No	1.052	0.49	Yes	NF	NF	
Catsup	NF	NF	No	0.387	0.21	No	NF	NF	
Brand soy	NF	NF	No	0.456	0.13	Yes	NF	0.11	
Soy	0.837	1.15	No	NF	NF	Yes	NF	NF	
Duck sauce	0.911	1.11	No	NF	NF	NF	NF	NF	
Mustard	0.745	32.14	No	NF	NF	Yes	NF	NF	

Table 8.5 Concentration of sorbate, benzoate and caffeine in various foods. The results are reported as the actual content found in the product. The agreed limit on sorbate is less than 3 mg/g. The legal limit of benzoate was less than 1 mg/g. The shaded entries are mislabeled. The levels shown in red are above the legal limit.

Similarly, several salad dressings listed the presence of benzoate, which was found to be present, but also significant levels of sorbate were found, which were not listed on the label. Both the lime juice and the ranch dressing slightly exceeded the legal limit for benzoate.

8.4 Summary of the quantification of benzoate, sorbate and caffeine

The calibration curve was generated with multiple standards at least $\pm 30\%$ both above and below the expected value using seven standards and a blank. The large number of standards was needed due to the wide range of concentrations expected. A total of six injections of each standard, produced RSDs of both retention time and area counts less than 1% with linear correlation coefficients of greater than 0.999, with small relative intercepts.

8.5 Chiral separations

When developing chiral compounds as potential drugs, each enantiomer needs to be synthesized or purified to contain less than 0.1% of each of the other enantiomer, to enable testing of the effects of each pure enantiomer, independently. Preferably, the major enantiomer is within the linear dynamic range (typically less than 2000 mAU) of the UV detector, while the minor contaminant (less than 0.1%) can be simultaneously measured with signal-to-noise greater than 10. If the major component

has a maximum absorbance of 1000 mAU (1 AU), then the minor component (0.1 %) should have a maximum absorbance of 1 mAU, and the noise should be less than 0.1 mAU peak-to-peak.

Chromatographic resolution should be at least 1.5 although significantly larger values are highly desirable, particularly if the minor component elutes after the major component. For such trace analysis, many consider a resolution of 3 to 6 as necessary, in order to quantify the minor component.

In a chiral separation of a racemate, the first peak is typically significantly higher and narrower than the second peak, although in terms of area, the peaks should be identical. The major and minor components each require development of separate calibration curves with multiple standards that bracket the expected results by at least $\pm 20\%$, and preferably $\pm 50\%$. At least five bracketing standards should be used for each curve. Each standard and sample should be injected at least six times. The calibration curves should be linear to at least 0.99. When signal-to-noise is greater than 100, the RSDs should approach or be better than 1 %.

In this example, the separation and quantification of the enantiomers of flurbiprofen over almost four orders of magnitude is demonstrated. Conditions were adjusted such that a 1 mg/mL solution produced two peaks, each approximately 1000 mAU high. Thus, a minor component representing 0.1 % of either enantiomer should be about 1 mAU high. The method consisted of 3 mL/min of 20 % methanol at 40 °C and 150 bar that separated the enantiomers of flurbiprofen using a 4.6 by 250 mm column, packed with 5- μm ChiralPak ADH. An excellent separation, with resolution greater than 5.5 and selectivity greater than 1.5, was obtained in less than 3 minutes.

The detector's signal beam was set to 280 nm with a bandwidth of 16 nm, and the reference beam was set to 360 nm with a bandwidth of 40 nm. The slit width was set to 8 nm. The filter was set to either 0.01 minutes (20 Hz) or 0.025 min (10 Hz).

The standard was progressively diluted to create additional standards for the calibration curves. Five bracketing standards (150, 125, 100, 75 and 50 %) were made for the high end calibration. For the low end calibration, five standards (0.015, 0.05, 0.1, 0.5 and 5 %) and a blank were used. Each standard was injected six times. Representative chromatograms of the six overlaid runs for 100, 0.05 and 0.015 % are presented in Figure 8.4.

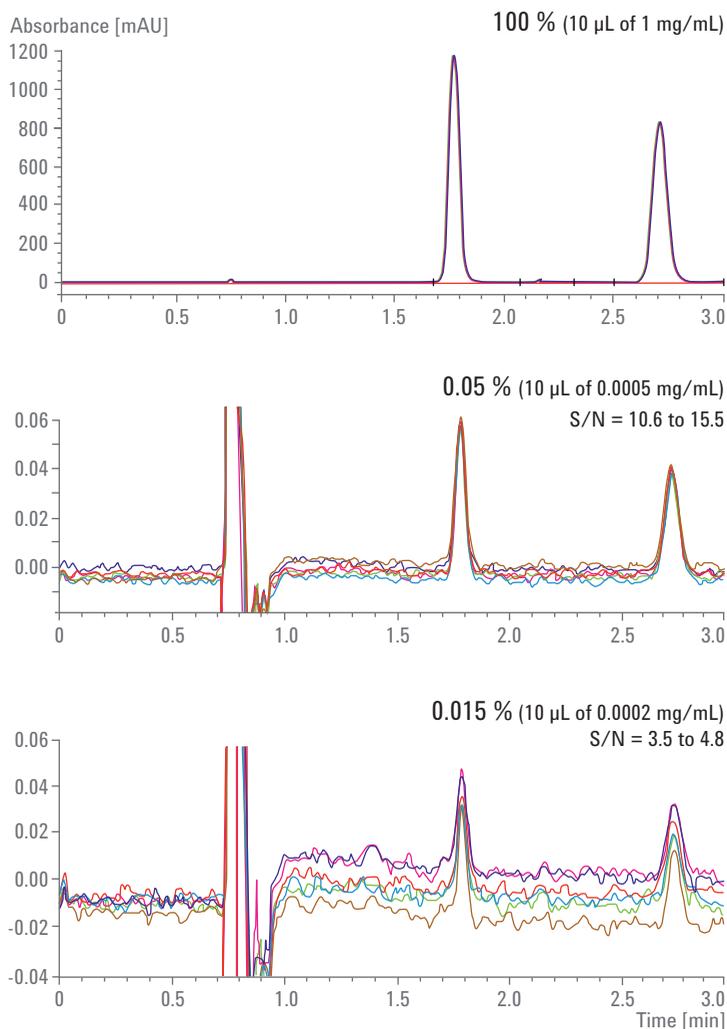


Figure 8.4 Chromatograms of racemic flurbiprofen at 100%, 0.05%, and 0.015% of the original value, separated on a 4.6 by 250 mm column packed with ChiraPak ADH, with 20% MeOH, 40 °C, 150 bar.

A similar set of separations was conducted by a different organization several thousand miles away, with a different instrument (a current Agilent HPLC instrument and an earlier Agilent HPLC instrument, both modified for SFC), and different operator, column, and chemicals. The only significant difference was the choice of 4 mL/min (against 3 mL/min) as the flow rate. Nevertheless, the quantitative results were similar, as indicated in Table 8.6.

	Flurbiprofen					
	User 1		User 2		Warfarin	ICH
	Peak 1	Peak 2	Peak 1	Peak 2		
Area reproducibility						
Area RSD at 100 %	–	–	0.9 %	0.9 %	0.27 %	≤ 1.0 %
Area RSD at 0.1 %	–	–	–	–	2.07 %	≤ 20 %
Area RSD at 0.05 %	3.36 %	3.87 %	4.6 %	5.0 %	–	None
Area RSD at 0.015 %	–	–	6.5 %	6.5 %	–	None
Area RSD at 0.01 %	–	–	–	–	14.2 %	None
Retention time reproducibility						
RT RSD at 100 %	–	–	0.1 %	0.1 %	0.46 %	≤ 1.0%
RT RSD at 0.05 %	–	–	0.1 %	0.1 %	–	–
RT RSD at 0.01 %	–	–	–	–	0.027 %	–
Sensitivity						
S/N at 0.05 %	17.7	18.2	15.9	10.6	57.6	> 10
S/N at 0.015 %	–	–	4.7	3.5	–	None
S/N at 0.01 %	–	–	–	–	7.9	None
LOQ (S/N = 10)	0.027 %	0.029 %	0.03 %	0.047 %	0.013 %	≤ 0.05 %
LOD (S/N = 3)	0.0084 %	0.0073 %	0.0095 %	0.0128 %	0.0038 %	≤ 0.017 %
S/N at 0.05 %	17.7	18.2	15.9	10.6	57.6	> 10
Linearity						
80 to 120 %	0.9990	0.9991	–	–	–	≤ 0.998
50 to 150 %	–	–	0.9985	0.998	–	–
0.05 to 5 %	1.0000	1.0000	0.9998	–	–	–
LOQ 120 %	–	–	–	–	0.99999	≤ 0.99

Table 8.6 The chiral separation of flurbiprofen on a 4.6 by 250 mm, 5- μ m ChiraPak ADH, 3 mL/min, 20 % MeOH, 40 °C, 150 bar. 13- μ L, 10-mm flow cell, 0.01-minute filter; 10- μ L injection.

The approximate limit of quantification (defined as the minimum signal retaining a signal-to-noise equal to 10), and limit of detection (defined as the minimum signal with signal-to-noise equal to 3), were found to be just under 0.015 and 0.005 %, as shown in Table 8.6. Both users obtained results within the guidelines of the International Committee for Harmonization (ICH), in terms of RSDs for area and retention time, linearity, signal-to-noise, limit of detection and limit of quantification, also shown in Table 8.6.

Also included in Table 8.6 are similar results from a separation of the enantiomers of warfarin on a ChiraCell ODH column with 30 % methanol containing dimethyl ethylamine as modifier, at 30 °C and 200 bar, indicating similar quantitative performance. Run time was under 4 minutes. Resolution was 3.8, and selectivity (α) was 2.1.

9.1 Pumping

9.1.1 Most SFC systems

In SFC, the CO₂ is usually supplied from steel cylinders containing liquefied CO₂ in contact with a gaseous headspace. The cylinder pressure is typically 55 to 85 bar near room temperature. Since the modifier is a normal liquid at room temperature and pressure, the mismatch in pressures requires separate pumps for the CO₂ and for the modifier. As a consequence, binary pumping systems are the norm.

Reciprocating pumps of the type used in HPLC and SFC have low compression ratios and cannot compress a low density gas. To pump CO₂ with such a pump, it must be liquefied. Most SFC systems use the liquid phase from the bottom of the cylinder to minimize power requirements. The liquid in the cylinder is in contact with a gaseous headspace. Any increase in temperature or decrease in pressure during a standard HPLC pump refill stroke would cause part of the liquid to vaporize, and the pump would create gaseous cavities in the liquid flow. Consequently, both the CO₂ and the CO₂ pump head are prechilled to 4 or 5 °C. Most SFC systems first use that pump to precompress the fluid from cylinder pressure to the column head pressure, and then accurately meter the CO₂ flow.

Chilling the pump head usually requires a bulky heat exchanger being bolted onto the front of the pump, making maintenance cumbersome. Further, a circulating chiller is often used to pump a cold glycol solution through the pump head heat exchanger. The tubing used to connect the chiller to the pump head heat exchanger requires extensive insulation to prevent water from the lab air condensing on the cold surfaces, depriving the chiller of much of its power.

Some pumps have a Peltier device mounted on the pump head, with a large finned heat exchanger with a fan. The fan draws lab air through the heat exchanger to carry away the heat. The cold side and the pump head need to be insulated to prevent excessive condensation of moisture out of the lab air.

CO₂ is far more compressible than normal liquids, even when chilled and liquefied. At supply cylinder pressures and typical chiller temperatures, adiabatic compressibility can exceed $1200 \times 10^{-6}/\text{bar}$, which is 8 to 27-times the compressibility of normal liquids. Fortunately, as the pressure is increased, the compressibility decreases, and by 400 bar, the compressibility of CO₂ is similar to some normal liquids. As a

consequence of this much higher compressibility, a much larger portion of the total available stroke length is used up simply compressing the fluid. This puts substantial demands on the pump and has often resulted in significant perturbations in pressure during compression.

9.1.2 Agilent analytical SFC system

The Agilent analytical SFC system is rather unusual compared to other SFC systems, as shown in Figure 9.1. All the aspects that make it an SFC system are contained in a separate conversion module. A largely unmodified HPLC makes up the rest of the system. In fact, the hardware can be used to rapidly switch back and forth between SFC and either normal or reversed phase HPLC in only a few minutes, as outlined in a later section. This rather detailed description of a specific SFC system is included since many of the comments below are relevant to this system, although in general they apply to most SFC systems.

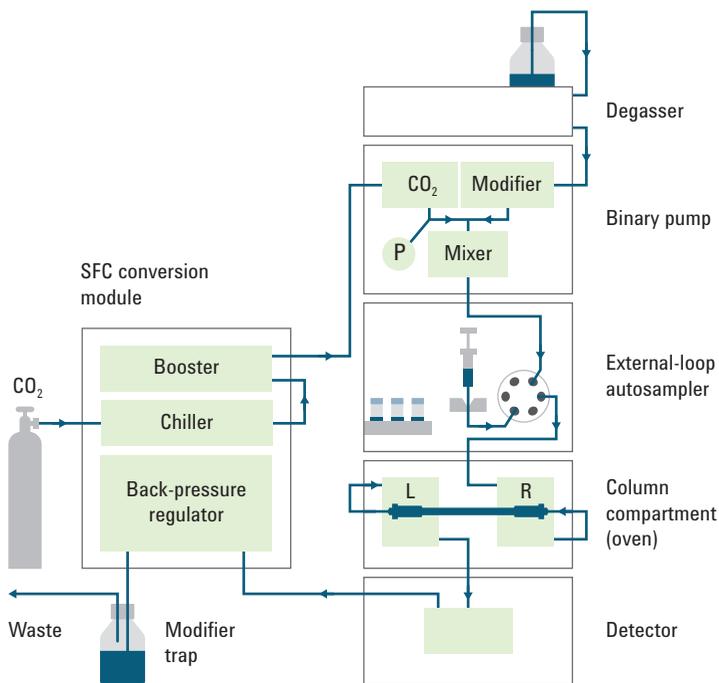


Figure 9.1 Schematic diagram of the Agilent analytical SFC system.

The vapor phase of CO₂ is supplied from a steel cylinder at room temperature, at a pressure of about 55 bar. The SFC conversion module contains a chiller that cools both the incoming fluid and the pump head of the booster pump. The booster pump increases the pressure of the CO₂ to 8 bar below the column head pressure. A binary pump meters and mixes the CO₂ and modifier with separate pumps. The autosampler is an external loop autosampler. The column compartment contains two heat exchangers. The right heat exchanger is used to precondition the mobile phase before it enters the column. The oven is a dead-air oven, which is good. The left heat exchanger is used to postcolumn condition the mobile phase temperature to near the detector cell temperature. The detector is a diode array detector (DAD) with a high-pressure flow cell. After passing through the DAD flow cell, the mobile phase is returned to the backpressure regulator in the SFC conversion module where it is allowed to drop to atmospheric pressure. After the pressure drop, the fluid breaks down to two phases. The liquid phase is collected in a trap, while the gaseous phase is vented into a fume hood.

The conversion module contains a powerful Peltier-based chiller plate mounted behind, and chilling the pump head. Unlike other SFC systems, the vapor phase from the CO₂ cylinder is drawn off, effectively distilling it just prior to use. This facilitates the use of low-cost industrial grades of CO₂, such as *beverage* grade. The chiller is powerful enough to liquefy the CO₂, and cool it to below -10 °C, even at 5 mL/min. The incoming fluid is prechilled by passing through a tube imbedded in the chiller plate, before entering the pump head. A sealed plastic cover shields the cold surface of the pump and chiller plate from the lab air. A small pump circulates a glycol solution through a heat exchanger on the hot side of the Peltier device to remove heat, which is dissipated through a radiator with a fan on the rear of the instrument. Thus, the tubing containing the glycol solution is never colder than lab air, eliminating condensation problems.

The A-side of the binary pump (not the booster) is used to meter the compressed CO₂ to the head of the column. The B-side of the binary pump is used to deliver the modifier. A pressure transducer mounted in the SFC conversion module is connected to the outlet of the binary pump through a T-piece and a pilot tube. This transducer monitors the outlet pressure of the binary pump. This signal is used to control the outlet pressure of the booster pump to just below the outlet pressure of the binary pump.

Neither side of the binary pump is chilled. The compressibility compensation of the A-side of the binary pump in the HPLC instrument, is set to zero. The B-side is set to Agilent's recommendation for the specific modifier.

Since the A-side does not compress the fluid, it acts solely to accurately meter the flow. This separation of compression and metering between two pumps in series dramatically reduces pressure noise. The conversion module also contains a low-noise, electronic backpressure regulator.

9.2 UV detector optimization

9.2.1 Software settings

It is fairly simple to optimize a DAD for low noise. The signal wavelength should be set to near an absorbance maximum. Since spectra are not often used in routine analysis, the bandwidth can be opened up to allow more light onto the photodiodes. The DAD averages the signals from all the diodes used, which tends to lower noise noticeably. However, if some of the diodes are at a wavelength of low absorbance, including them in the averaging is a poor choice. Either the wavelength should be changed or the bandwidth narrowed.

Many detectors also allow the adjustment of the width of the slit entering the monochromator. The slit is usually set to the same value as the bandwidth of the signal beam. However, if spectra are required, the slit should be reset to its lowest value of 1 or 2 nm, to avoid blurring fine structures in the spectra.

A reference wavelength should be used whenever possible. Some noise and drift are due to fluctuations in the lamps that occur at all wavelengths. By using the ratio of the signal to the reference wavelength, such noise can largely be eliminated. The reference wavelength should be set as close to the signal wavelength as possible, without allowing significant absorbance by the sample. The reference bandwidth is often set rather wide at 80 nm but narrower values such as 40 nm or even lower can be used with only minimal increases in noise.

Situations should be avoided where the signal bandwidth involves sampling light mostly from one lamp, while the reference is sampling light from the other lamp, since this may increase noise. Such a situation is most likely when the sample absorbs at relatively high UV wavelengths, and the reference is set to even higher wavelengths.

A wavelength of 205 or 210 nm is regarded as enabling near universal detection since most organics absorb at least a little light in that region. CO₂ is completely transparent to below 190 nm. The UV cut-off for methanol is about 205 nm, which facilitates operation below 210 nm with minimal noise. Both ethanol and isopropanol have a slightly higher UV cut-off at about 210 nm. Although seldom recommended, acetonitrile has a UV cut-off of 195 nm, enabling detection below 200 nm.

Additives are often acids or bases that generally absorb out to about 230 nm, which can limit the ability to detect compounds with weak chromophores. A high-purity modifier should always be used to avoid absorbance at higher wavelengths.

9.2.2 Data rate

UV noise increases 10-fold from 2.5 to 80-Hz data rate, as shown in the upper panel of Figure 9.2. Too low data rate decreases noise but also degrades peak shape and resolution. Too high data rate results in excessive noise and loss of sensitivity. Clearly, the filter should be set to just pass the fastest expected peak from any given column. Agilent chromatography data systems list filter settings as peak width in minutes, response time in seconds, and frequency in Hertz. The integrator reports peak widths in minutes. The filter should be set to the next higher speed. You can find the best filter setting empirically. After collecting a chromatogram with an initial filter setting, decrease the filtering by one setting (less filtering) and repeat the chromatogram. If the reported peak width does not change, the original setting was probably correct. If the reported peak width does change, decrease the setting further and repeat the chromatogram. Keep lowering the filter until the reported peak width is unchanged.

With older methods, using 4.6 by 250-mm columns with 5- μm particles, a filter setting of 2.5 Hz was appropriate. With 3 by 100-mm columns with 1.8- μm particles, a filter setting of 40 Hz is usually adequate.

The actual noise measured is variable, depending on the age of the lamp and the nature of the cell, among other factors discussed in the next few sections. In the lower panel of Figure 9.2, an older 6-mm, 1.7- μL cylindrical flow cell is compared to a 3-mm, 2- μL conical flow cell, both with a nearly new lamp. Noise was almost a linear function of frequency. The noise with the 3-mm cell at 80 Hz was nearly an order of magnitude lower than the Berger Instruments DAD from 1995 with a 2.5-Hz filter.

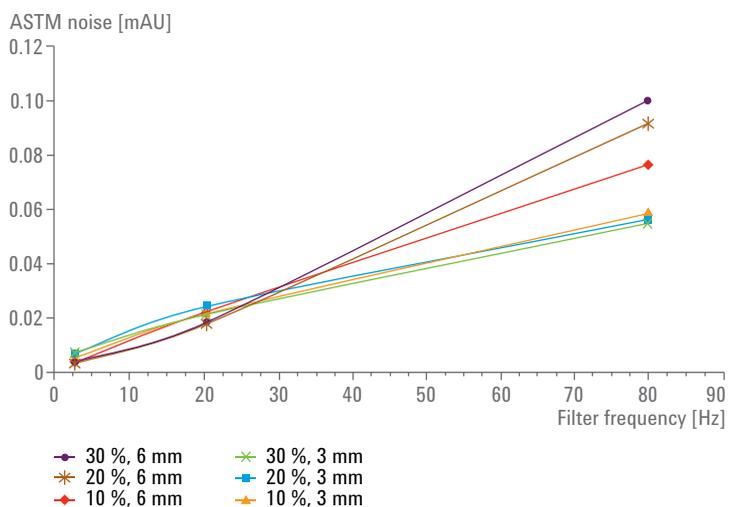
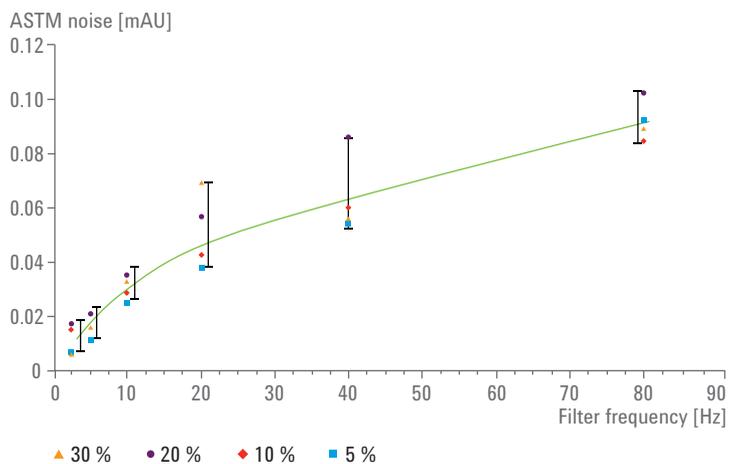


Figure 9.2 The effect of filter frequency on optimized ASTM noise with 2.5 mL/min. Data was collected at 5 to 30 % MeOH, 150-bar outlet pressure using; (upper panel) 3-mm, 2- μ L flow cell with a conical flow path and with an older lamp, and (lower panel) with both the 3-mm, 2- μ L flow cell, and a 6-mm, 1.7- μ L flow cell with cylindrical flow path and with a new lamp. The noise with the 3-mm cell nearly doubled as the lamp aged.

9.2.3 Refractive index

UV detectors designed for HPLC have optics with a focal length that depends in part on the refractive index (RI) of the fluid passing through the cell. RI is a function of the density of fluids. The RI of water at 25 °C is 1.333. The RI of most other common HPLC solvents lies between 1.328 and 1.375. Detector designers assume an RI within this general range. The density and the RI of such solvents changes little with pressure.

As shown previously, in Figure 2.1 and Figure 2.3, the density of both pure CO₂ and modified CO₂ are dependent on temperature and pressure^{114–116}. Similarly, the RI is a strong function of both temperature and pressure as shown for pure CO₂ in Figure 9.3 and Figure 9.4. The minimum RI value for pure CO₂ is 1.08, which is far from the RI of any of the normal liquid solvents used in HPLC. The maximum RI calculated for CO₂ was about 1.24. The addition of a modifier to the CO₂ is likely to increase the RI of the mixture toward the values for the pure solvents. For example, the density of methanol/CO₂ mixtures is always higher than the density of either pure fluid at the same temperature. Thus, the changes in RI caused by changes in temperature, pressure or composition can cause large baseline shifts and potentially increase noise due to changes in the focal length of the cell.

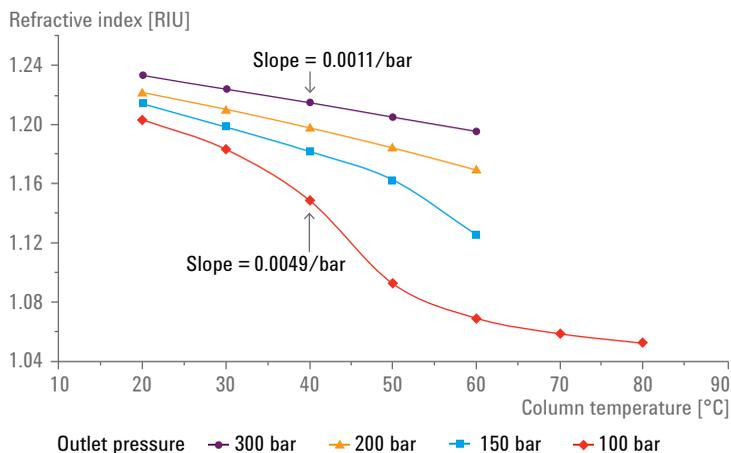


Figure 9.3 Effect of temperature on the refractive index of pure CO₂ at four different pressures. The same small changes in temperature at 100 bar result in much larger changes in RI than at 300 bar.

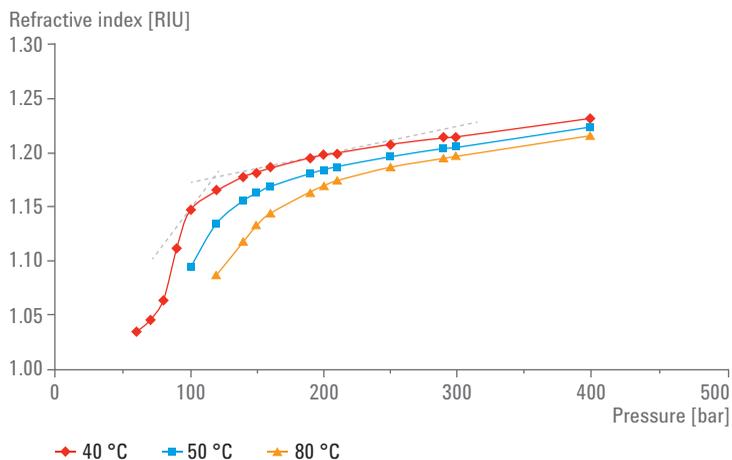


Figure 9.4 Refractive index of pure CO₂ as a function of pressure at three temperatures: 40 °C; 50 °C; 80 °C. The dashed lines indicate the change in slope between 100 and 200 bar. The same small changes in pressure at 100 bar result in much larger changes in RI than at 200 bar.

9.2.3.1 UV flow-cell temperature and noise

SFC is often conducted at temperatures significantly different from room temperatures. Fluid leaving the column can be many degrees warmer or cooler than the flow cell in the detector, which is heated by its internal electronics. When the fluid enters the flow cell the temperature must equilibrate, potentially causing large changes in RI within the cell. This causes changes in the focal length of the optics. Light hitting the wall of the cell can be reflected away from the slit, causing a variation in the light entering the monochromator. This effect generates a varying baseline signal, which is UV noise.

One of the heat exchangers in the column compartment can be used to condition the postcolumn mobile phase temperature to match the actual cell temperature, eliminating most noise associated with thermal differences. A schematic is presented in Figure 9.5.

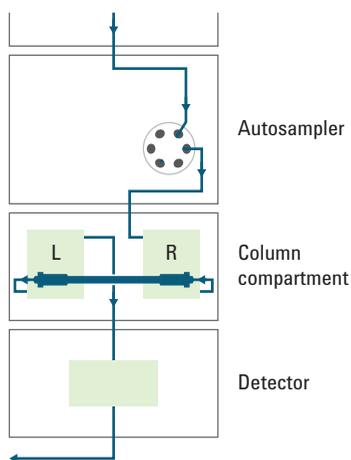


Figure 9.5 Schematic diagram showing the plumbing arrangement for the column compartment that allows the right heat exchanger to precondition the mobile phase temperature before it enters the column, and use the left heat exchanger to postcolumn condition the mobile phase temperature to the detector flow cell temperature.

Many modern UV detector cells have a conically shaped aperture for light to pass through. In HPLC, this shape has been shown to be important in minimizing noise due to RI effects caused by short-term variations in temperature. In SFC, this may not be as important. In fact, one 10-mm path length cell with the conical flow path produced excessive noise when the temperature of the fluid entering the flow cell was more than a few degrees different from the flow cell temperature, as shown with the upper curves in Figure 9.6. The curves were obtained at 5, 10, 20 and 30 % methanol in CO₂ at 2.5 mL/min and 150-bar outlet pressure. Both heat exchangers were varied between 20 and 70 °C. However, when the right heat exchanger was varied between 20 and 70 °C, while the left heat exchanger was maintained (optimized) at the cell temperature, virtually all the thermally induced noise disappeared as shown by the lower curves in Figure 9.6.

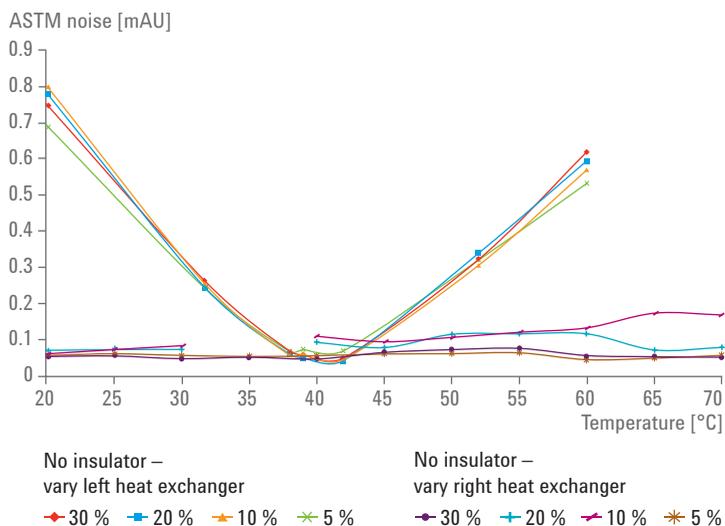


Figure 9.6 ASTM noise measured at four modifier concentrations using an older 10-mm, 13- μ L flow cell with conical flow path and without a thermal insulator. The temperature of the effluent from the left heat exchanger was varied between 20 and 80 °C just before it entered the flow cell. The mismatch in temperature caused excessive noise as seen in the upper set of curves. The experiment was repeated except the right heat exchanger was used to vary the column temperature between 20 and 70 °C, while the left heat exchanger temperature was constant at 41 °C. Virtually all the temperature-induced noise disappeared as shown with the near horizontal curves at the bottom of the figure.

Surprisingly, a nearly identical flow cell with a conical flow path but with a ceramic insulator between the cell and the optical bench produced almost no thermally induced noise. The nonoptimized noise from this insulated cell is shown as the lower, nearly horizontal curves in Figure 9.7. The nonoptimized noise curves from the uninsulated flow cell (top curve, Figure 9.6) are included for comparison. In this instance, the insulator appeared to be more important than the conical shape. Many SFC systems as little as a few years old use the uninsulated cell as standard. Immediate improvement in noise could be expected by switching to the newer insulated cell.

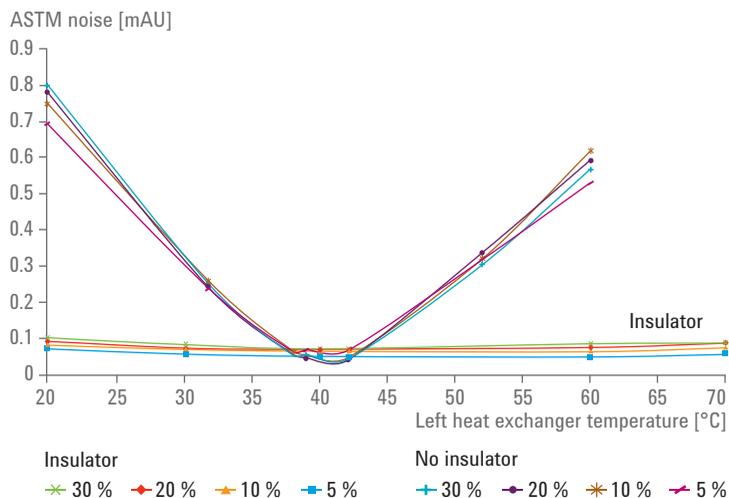


Figure 9.7 A nearly identical cell as in Figure 9.6 but with a ceramic insulator thermally isolating the cell from the optical bench produced dramatically less noise, even when the left heat exchanger temperature was varied between 20 and 70 °C, as can be seen with the lower, nearly horizontal curves. The upper curves from Figure 9.6 are included for comparison.

The fact that the conical shape was not the determining factor in the noise level may be due to the fact that the RI of CO₂ and presumably mixtures of CO₂ with modifiers, is much lower than the RI of the normal liquids used in HPLC, and therefore bends light much less. The flow cells were originally designed for HPLC, where the conical shape was apparently more important.

On the other hand, a 6-mm, 1.7-μL flow cell with a cylindrical flow path has been available for many years and shows similar, but not quite as severe, performance to the uninsulated 10-mm cell. This was true regardless of the identity of the modifier, as shown in Figure 9.8. Two chromatograms collected with this flow cell are shown in Figure 9.9. In the upper chromatogram, the left heat exchanger was set to 30 °C, while the flow cell temperature was 41 °C. In the lower chromatogram, the left heat exchanger was optimized. Note the small peaks that could be distinguished in the lower chromatogram, due to the lower noise.

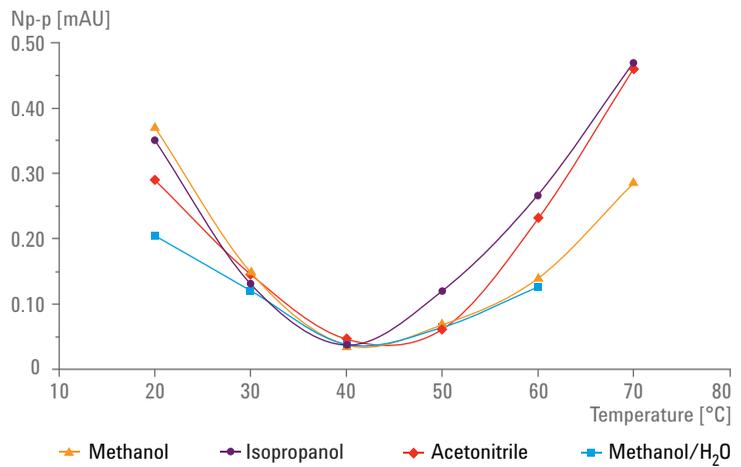


Figure 9.8 Another older flow cell with a 6-mm path length and 1.7- μ L volume, but a cylindrical flow path, was similarly evaluated using several modifiers. The results are similar regardless of the modifier used. This cell was only half as noisy as the 10-mm uninsulated cell, but much noisier than the 10-mm insulated cell. Using the left heat exchanger to optimize performance produced the optimized results in Figure 9.2.

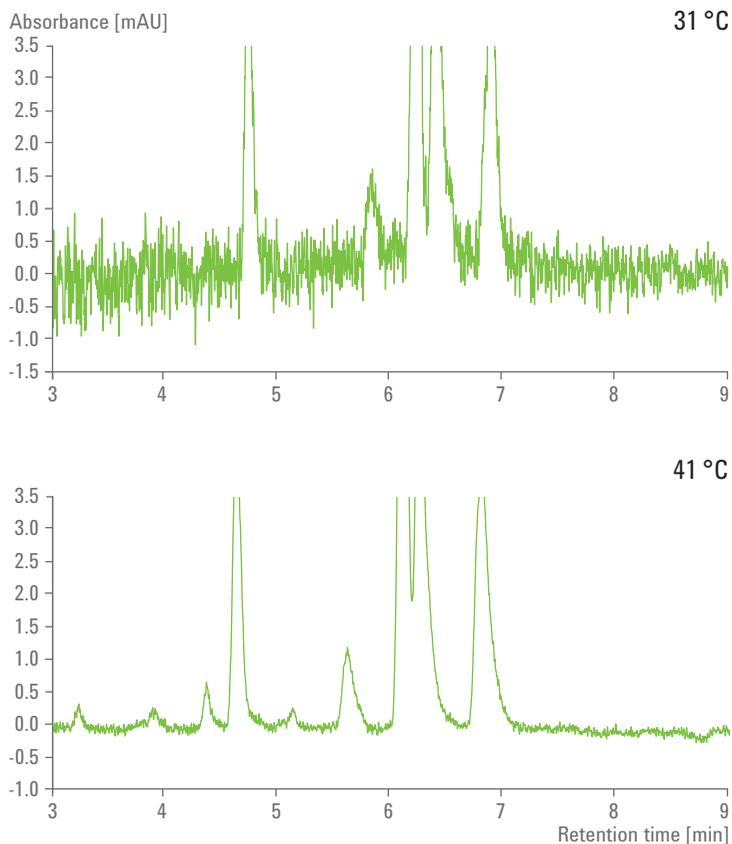


Figure 9.9 Chromatograms collected with the 6-mm flow cell used in Figure 9.8. The upper curve was collected with the left heat exchanger set to 30 °C. The lower curve was collected with an optimized left heat exchanger of 41 °C, 100-bar outlet pressure.

Confusingly, an uninsulated 3-mm path length cell with a conical flow path, but without the insulation, showed little susceptibility to temperature induced RI effects, similar to the 10-mm insulated cell. However, it was still necessary to adjust the left heat exchanger temperature to the flow cell temperature to achieve minimum noise as shown at 40 Hz in Figure 9.10. Insulating that cell with a ceramic spacer did not improve noise further. Surprisingly this 3-mm cell produced only 87 % of the noise of the longer, much larger volume (13 versus 2- μ L) cell.

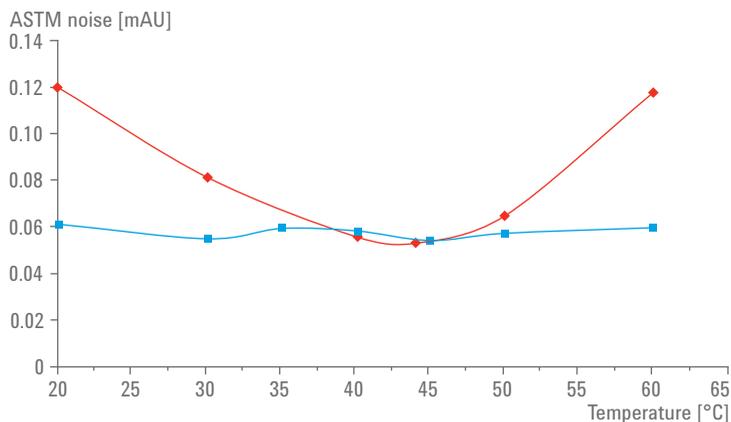


Figure 9.10 Yet another flow cell, with a 3-mm path length, 2- μ L volume, uninsulated, with a conical flow path produced much less noise than the cell in Figure 9.8. Using the left heat exchanger to optimize further, eliminated noise.

9.2.3.2 Column outlet pressure and noise

All electronic backpressure regulators (BPR) are continuously dithering their control electronics around the set point. This results in small variations in the outlet pressure. If not tightly controlled, this dithering can lead to significant noise. The Agilent analytical SFC system contains a state-of-the-art BPR for SFC with a pressure noise of as little as ± 0.05 bar at 150 bar. This is greater than 10-times better than previous generations of electronic BPRs for SFC.

As mentioned previously, the RI of CO_2 is a strong function of its density, which in turn depends on its temperature, pressure and composition. Despite the low-pressure fluctuations obtained with the Agilent analytical SFC system, some pressure-related noise can be seen under some conditions. It should be noted that probably 80 % of all reports on packed column SFC from before 1990 to after 2008 used 40 °C with 100-bar outlet pressure as the primary set points. In Figure 9.4, it was shown that at 40 °C and 100 bar the RI of CO_2 changes steeply with small changes in pressure. This is an inherent characteristic of the fluid. In past years this was largely ignored, since other noise sources were larger and tended to obscure the effect of pressure-induced noise.

The slope of RI against pressure decreases fairly rapidly above approximately 120 bar. A plot of UV detector noise against outlet pressure is shown in Figure 9.11. The outlet pressure was changed from 100 to 200 bar in several stages at 40 °C. At 100 bar, the noise was about 0.11 mAU with a filter setting of 0.05 min (5 Hz). At 120 bar, the noise dropped by over 50 % to about 0.04 mAU. At 140 bar, the noise fell

another 50 % to about 0.02 mAU. In the author's laboratory the default settings for outlet pressure and temperature have been 150 bar and 50 °C for the last 4 to 5 years. For the lowest possible noise, an outlet pressure of 200 bar is recommended.

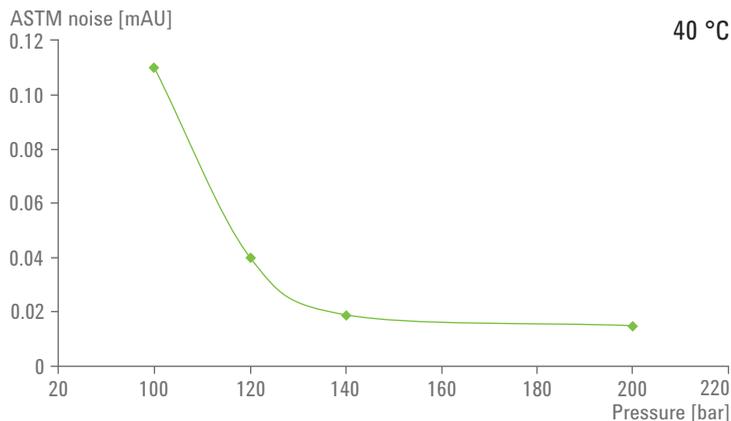


Figure 9.11 Effect of outlet pressure on Noise. At 40 °C, the refractive index changes much more with small changes in pressure at low column outlet pressures such as 100 bar, than at high outlet pressures such as 200 bar. Conditions: 3 mL/min of 4 % MeOH, 40 °C, filter set at greater than 0.05 minutes.

With modified mobile phases, especially with modifier concentrations much above 10%, pressure becomes a secondary control variable with little impact on either selectivity or retention. Since the viscosity of the fluids is so low, the pressure drops occurring at optimum flow rates, even using sub-2- μm particles, are modest, so a set point of 200 bar for the outlet pressure has only minor consequences. The column head pressure should seldom exceed 400 bar, although the system is capable of operating to 600 bar.

There are instances such as with mixtures of similar nonpolar compounds where it is desirable to operate at pressures even below 100 bar to maximize resolution between closely related compounds. Increasing the temperature tends to flatten out the curves of RI against pressure, which can help. Under such cases, there is a trade-off between sensitivity and resolution since the RI is an inherent property of the fluid.

9.2.3.3

Low modifier concentration and noise

In SFC, the modifier concentration seldom exceeds 50 %, except during rapid screening runs. Since the range of modifier concentration is lower than in HPLC, concentrations well below 10 % modifier are often used. However, at low flow rates and low modifier concentration the modifier pump may not cycle more than once a minute or even slower. At 2 mL/min and 5 % modifier, the modifier pump is delivering 100 μ L/min. The Agilent analytical SFC system has a variable stroke length. At that flow rate, the pump stroke is probably 20 μ L. Thus, the pump makes five strokes per minute.

When the fluid is being compressed to working pressure, no flow leaves the primary piston, while the secondary piston is withdrawing fluid from downstream. A downstream check-valve between the B pump and the mixing point is intended to prevent this backflow from reaching the mixing point.

After the compression stroke, the modifier pump injects additional modifier to make up for the total shortfall. The flow and composition are accurate but with a periodic perturbation of the baseline. The mixer must attempt to average out this slow oscillation in modifier flow, although the variation may occur only once a minute or slower.

The primary mixer was designed for mainstream HPLC, involving at least 1 mL/min with 5 % modifier or greater. At low flow or modifier concentration, the standard mixer has difficulty completely blending out these perturbations. Since the modifier has a different RI compared to the CO₂, any oscillation in modifier concentration, particularly at low concentrations, creates another lower frequency form of noise at the modifier pump frequency.

A plot of noise against modifier concentration is presented in Figure 9.12. At 2 mL/min, 40 °C and 200 bar, the worst noise was found at 1 % methanol, where the pump was operating at only one stroke per minute. Any need to operate at such low modifier concentrations is the result of using a modifier that is too strong for the solutes.

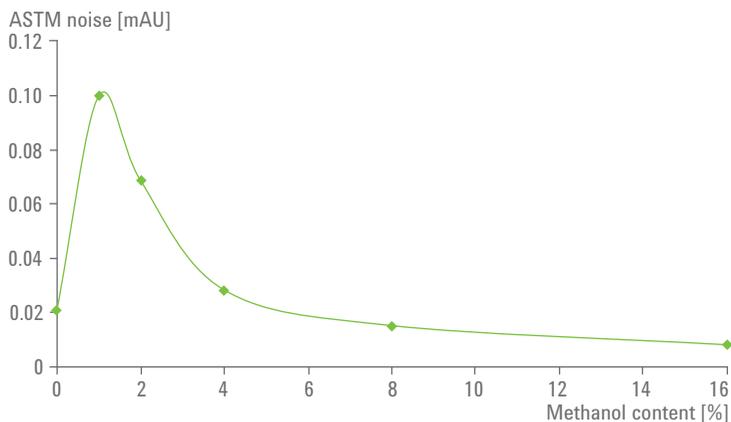


Figure 9.12 Noise induced by modifier pump at low modifier concentrations. The modifier pump is operating at low frequency and perturbations in composition at that low frequency are not being adequately averaged by the standard mixer. Conditions: 2 mL/min, 40 °C, 200 bar. Filter greater than 0.1 minutes.

There are newer versions of mixers that provide variable path lengths such as the Agilent Jet Weaver mixer, but with much lower volume.

Alternately, avoid low modifier concentrations by decreasing the solvent strength, that is, the polarity, of the modifier. With a weaker modifier, the compounds are more retained, requiring higher concentrations. Ethanol decreases solvent strength significantly, compared to methanol, as pointed out in Chapter 2 “The Mobile Phase”. Solvent strength is further decreased with isopropanol. Many other organic solvents are also usable as modifiers that further decrease solvent strength, increase retention, or change selectivity. However, some solvents such as chlorinated solvents have negative environmental impact and are avoided.

9.3 The doubling gradient

There are further implications regarding the *rule of thumb* that doubling the modifier concentration halves retention. The first small additions have a disproportionate effect on retention but at higher modifier concentrations, small changes have minimal effects. Nevertheless, modifier concentration is most often programmed linearly. Thus, at the beginning of a gradient, peaks are pushed together and at the end of the gradient they are broadened. If the modifier concentration is initially changed only gradually, but the rate of change is progressively increased during the run, the early eluters will be better resolved while the late eluters will be sped up. Such gradients were proposed more than 20 years ago but apparently have not been widely embraced.

The column hold-up time, t_0 , (retention time of an unretained peak) is a convenient time base for translating methods between columns of different dimensions. If you start at the lowest feasible percent modifier, some compound might elute in the first few column hold-up times after t_0 . With a properly chosen modifier, most solutes in a complex mixture will be easily retained at the initial conditions. Holding the initial concentration for one or more column hold-up times produces the maximum resolution under the weakest elution conditions. Doubling the concentration during each subsequent multiple of t_0 results in all peaks emerging with about equal peak widths, which is similar to an isocratic peak with k approximately equal to 1 to 2. If each doubling of the concentration reduces retention by a factor of 2, starting from 2 % and progressing to 64 % should change retention by about 64 %. This type of gradient can be rapid yet degrade resolution no worse than about 40 % from optimized isocratic separations.

9.4 Autosampler considerations

9.4.1 Variable-loop versus fixed-loop

Many current autosampler designs use the variable-loop or flow-through design concept, but SFC is incompatible with this approach. In a variable-loop autosampler, a needle is connected to a high-pressure metering device (such as a syringe) with a piece of flexible stainless-steel tubing that acts as the sample loop. During sample loading, the mobile phase is diverted directly from the pump to the column by switching an injection valve into bypass (load) mode. Sample can then be withdrawn from a vial at atmospheric pressure into the needle and loop. After withdrawing the sample, the needle is pushed into a high-pressure needle seat, and the injection valve is switched (inject), diverting the mobile phase from the pump through the metering device, loop, needle and seat, then through the valve onto the head of the column. The needle and the sample loop can remain in the inject position to be flushed by mobile phase in order to minimize carryover.

In HPLC, this approach has many beneficial aspects, since the mobile phase is essentially an incompressible liquid. Switching the valve to the bypass position isolates the metering device, loop, and needle from the flowing stream and connects the needle seat to waste. The fluid in this isolated section immediately expands minimally and the pressure drops to atmospheric. The metering device, loop and needle remain filled with the liquid mobile phase.

In SFC, the mobile phase behaves like a *compressed gas*. When the valve is switched to the bypass position, the contents of the metering device, connecting tubing (loop), needle and needle seat all expand up

to 500-times their compressed volume, and rapidly vent through the waste port of the valve to ambient. After this expansion, the whole injection system is filled with a low density gas. Attempting to withdraw the next sample results in the cavitation of the metering device, which results in it being unable to withdraw any further samples from the sample containers.

Variable-loop autosamplers need to be modified for use in SFC by converting them to fixed-loop operation. An external fixed-volume loop is connected between opposite ports of a 2-position/6-port valve. The sample is withdrawn from a vial, and then pushed through the loop with the same high pressure metering device. When the injection valve is turned, only the loop and rotor grooves experience the high pressure of the system.

When using an injector program, the needle should be left in the needle seat, and withdrawn only after the valve returns to the bypass position. If the needle is prematurely withdrawn from the needle seat before the valve is switched, the contents of the metering device (about 200 μ L), the loop and the needle all rapidly vent as a highly compressed gas into the interior of the sample compartment, contaminating all the surfaces with potentially harmful mobile phase components such as some additives. If the needle is left in the needle seat, all this fluid is vented out the waste line. The waste line should always be connected to a liquid trap and the vapor phase should be diverted into a fume hood. Before the next injection can be made, a wash pump mounted in the SFC module washes the metering pump, loop and needle with fresh solvent.

9.4.2 Carryover

The SFC control module in the Agilent analytical SFC system contains a wash pump that thoroughly washes the injection valve, syringe, connecting tubing, and needle between injections. This usually results in exceptionally low carryover. An Example is shown in Figure 9.13. With the highly concentrated sample, the peak with a reported area of greater than 17,500 was off-scale at greater than 2500 mAU due to detector saturation. A subsequent blank injection produced a peak with area reported as 0.88. Carryover in terms of area was about 0.005 %.

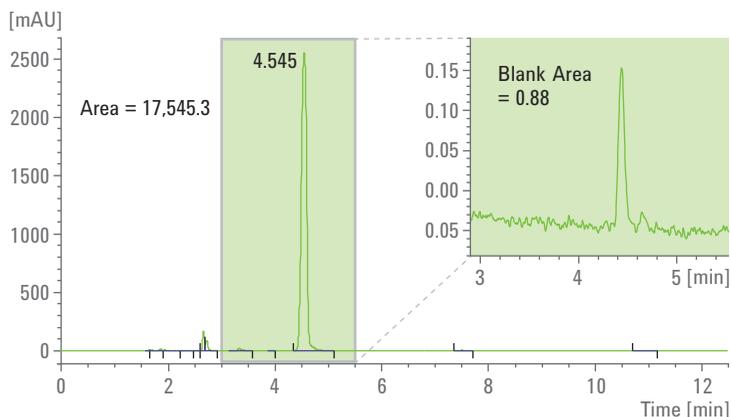


Figure 9.13 Carryover of caffeine. A highly concentrated sample was injected followed by a MeOH blank. Between injections the syringe, connector tubing, needle, needle seat, valve and external loop were washed with approximately 0.5 mL MeOH. Carryover was 0.005 %. Conditions: 2 mL/min of 5 % MeOH in CO₂, 40 °C, 200 bar. Column: 4.6 by 250 mm, 5 μm, RX-SIL. Filter greater than 0.05 minutes, 15 μL into a 5-μL loop with 1.5-μL air bubbles on each side of the sample plug.

9.4.3 Reproducibility

In regulated environments, reproducibility of both retention time and peak area are required to be better than ± 1 % RSD, when signal-to-noise is greater than or equal to 100. These levels of reproducibility can be achieved easily in SFC, provided full-loop injections are made and the loop is overfilled at least several times. For example, with a 1.25-μL loop, an injection volume of 5 to 7 μL should be more than adequate. It is possible to load the sample such that only the middle, undiluted part of the sample plug is in the external loop.

Retention-time reproducibility is usually better than 1 % in modern SFC. However, area reproducibility is also dependent on signal-to-noise. To achieve less than 1 % area reproducibility, the uncertainty in the signal can be no better than about ± 1 %, if the signal-to-noise is 100. However, with low noise such as 0.01 to 0.02 mAU, a signal-to-noise of 100 can be achieved with a peak of only 1 to 2 mAU high.

Two sets of reproducibility measurements ($n = 10$) are presented. In the first, a high modifier concentration and high pressure were used, which produces among the best reproducibility. The first example, presented in Table 9.1, shows the retention time and area reproducibility of caffeine, theophylline and theobromine. Retention-time reproducibility averaged 0.033 % RSD. Area reproducibility, with a signal-to-noise less than 300 to about 1400, was between 0.1 and 0.29 % RSD.

Caffeine		Theophylline		Theobromine		
RT	Area	RT	Area	RT	Area	
1.420	1471.8	1.895	2305.2	2.198	273.9	
1.419	1471.9	1.895	2309.8	2.197	275.7	
1.420	1472.5	1.898	2307.9	2.198	275.8	
1.419	1472.3	1.895	2308.7	2.197	273.3	
1.420	1473.1	1.895	2308.8	2.197	274.5	
1.419	1472.4	1.895	2308.5	2.198	274.2	
1.420	1474.8	1.895	2307.0	2.198	274.0	
1.420	1475.2	1.895	2312.8	2.198	273.9	
1.420	1474.9	1.895	2309.0	2.195	274.3	
1.420	1475.3	1.895	2312.9	2.195	274.1	
Mean	1.4197	1473.4	1.8951	2308.8	2.1985	274.37
SD	0.0005	1.4021	0.0003	2.3203	0.001	0.7524
% RSD	0.04	0.10	0.02	0.10	0.05	0.27

Table 9.1 Reproducibility of caffeine, theophylline and theobromine under isocratic conditions with high flow rate, high % modifier, and high pressure. Conditions: 40 % MeOH, 200 bar, 4 ml/min, 40 °C. Column: 4.6 by 250 mm, 5 µm, RX-SIL.

The second set of reproducibility measurements were collected using a 10 % per minute gradient in the separation of a mixture of profens and xanthenes. Modifier composition was held constant at 2 % for 1 minute, then 10 % per minute to 42 %, at 3 mL/min, 30 °C and 200 bar, using a 4.6 by 250 mm, 5-µm silica column. The retention-time RSDs averaged 0.11. The area RSDs averaged 0.38, as seen in Table 9.2.

Ibuprofen		Caffeine		Theophylline		Ketoprofen		Theobromine		
RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	
2.850	507.02	3.372	440.32	4.497	1183.3	4.883	1875.0	5.777	383.57	
2.849	503.98	3.372	439.38	4.493	1173.9	4.879	1875.1	5.772	385.08	
2.848	502.80	3.372	438.88	4.491	1171.9	4.878	1873.4	5.789	384.98	
2.850	507.98	3.373	442.80	4.489	1174.7	4.874	1875.7	5.785	384.74	
2.851	507.98	3.375	441.18	4.487	1171.3	4.872	1873.8	5.782	384.87	
2.851	508.22	3.375	442.50	4.485	1175.7	4.870	1878.3	5.759	381.78	
2.853	508.48	3.377	444.01	4.485	1182.2	4.871	1890.0	5.758	385.89	
2.854	508.00	3.378	441.28	4.483	1170.0	4.888	1870.0	5.753	383.18	
2.852	507.81	3.377	442.82	4.481	1172.7	4.888	1877.2	5.752	382.11	
2.852	508.42	3.377	443.08	4.480	1178.3	4.884	1880.9	5.749	385.25	
Mean	2.851	507.08	3.3748	441.4	4.4871	1175.2	4.8723	1878.9	5.7814	384.12
SD	0.0018	2.0002	0.0024	2.109	0.0054	4.4259	0.0059	5.4424	0.0093	1.3978
% RSD	0.07	0.39	0.07	0.48	0.12	0.38	0.13	0.29	0.18	0.38

Table 9.2 Reproducibility with a 10 % per minute gradient: 3 mL/min of 2 % MeOH for 1 minute then 10 % per minute to 42 %, then hold, 40 °C, 200 bar on a 4.6 by 250 mm, 5 μ m, RX-SIL column.

9.4.3.1 Partial-loop injections

With partial-loop injections, retention-time reproducibility was similar to full-loop injections, but area reproducibility degraded somewhat. Linearity was excellent. At greater than 1.5 μ L on a 5- μ L loop, area RSD was about 2 %. At less than 1 μ L, the RSDs are less than 10 %.

9.4.4 Injection volume and the effects of strong sample solvent

This is a neglected aspect of SFC that needs far more attention. Samples are often dissolved in a relatively strong solvent such as methanol. This potentially causes a problem, which is also present in HPLC. The mobile phase in SFC can be rather nonpolar, particularly when the modifier concentration is low. Injecting a sample dissolved in a strong (polar) solvent into a low concentration of the same solvent, used as modifier, results in a situation where at least the center of the sample plug is much more polar than the bulk mobile phase. Solute molecules in the center of this band experience a stronger solvent than the rest of the mobile phase, and are subsequently less retained than solute molecules on the edges of the band. This results in peak broadening, tailing, and loss of resolution between closely retained peaks.

Increasing the injection volume often accentuates the effect. An example using the separation of four corticosteroids, dissolved in methanol, on a 4.6-mm id column using 8 % methanol as modifier, was shown in Figure 2.5. Pure methanol as sample solvent is much more polar (a much stronger solvent) than 8 % methanol in CO₂.

You would expect a linear relationship between the injection volume of a standard and the peak height of the resulting peaks, if there was no broadening due to the strong sample solvent effect. However, the peak heights of the solutes were found to be a linear function of injection volume up to only about 6 to 7 μ L, as shown in Figure 9.14. At higher injection volumes, the peak height rolled off significantly. Similarly, a plot of peak width at half height also shows a break in the same vicinity of injection volumes, although the break is less clear-cut, as shown in Figure 9.15. Finally, a plot of peak area against injection volume was linear, as shown in Figure 9.16, indicating that the appropriate amounts were being injected but progressively broadened at higher injection volumes.

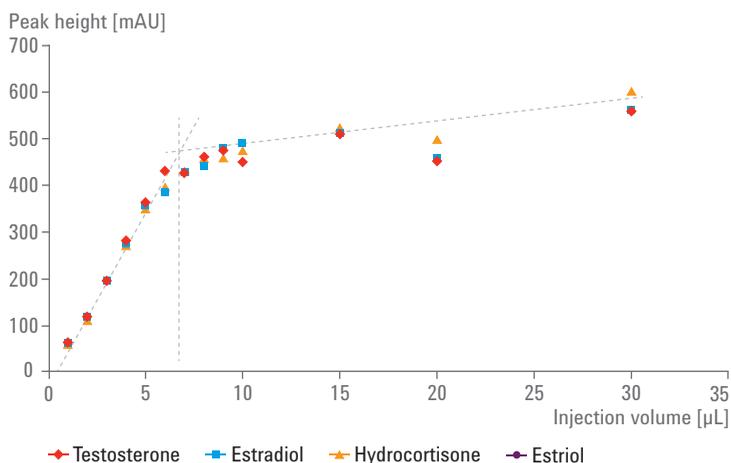


Figure 9.14 The effect of strong sample solvent. The effect of injection volume on the peak height of testosterone, estradiol, hydrocortisone and estriol dissolved in MeOH, using 8 % MeOH in CO₂ as the mobile phase at 40 °C, 100-bar outlet pressure. Column: 4.6 by 150 mm, 5 μ m, bare silica. Height rolls off above about 8 μ L.

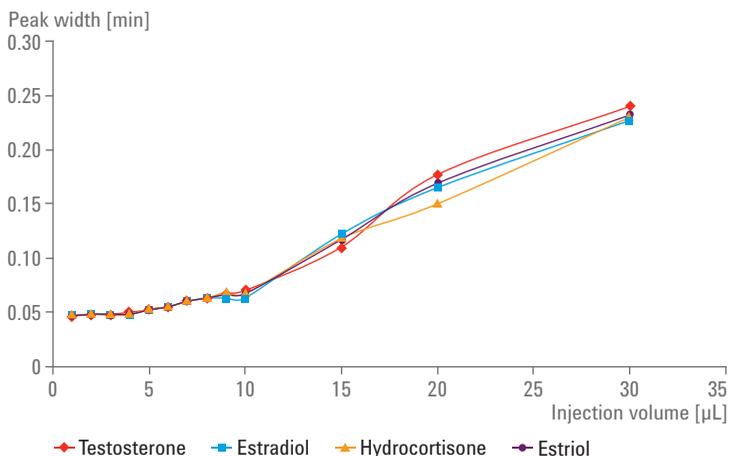


Figure 9.15 The effect of injection volume on the peak width of testosterone, estradiol, hydrocortisone and estriol dissolved in MeOH, using 8 % MeOH in CO_2 as the mobile phase at 40 °C, 100-bar outlet pressure. Column: 4.6 by 150 mm, 5 μm , bare silica. Peak width starts to degrade about 6- μL injection volume.

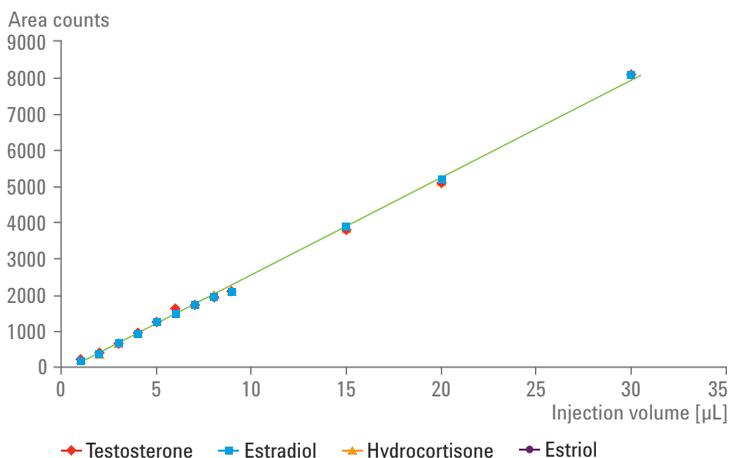


Figure 9.16 The effect of injection volume on the peak area of testosterone, estradiol, hydrocortisone and estriol dissolved in MeOH, using 8 % MeOH in CO_2 as the mobile phase at 40 °C, 100-bar outlet pressure. Column: 4.6 by 150 mm, 5 μm , bare silica. Peak area is a linear function of the amount injected.

Samples should never be dissolved in a solvent more polar than the modifier in use. For example, the sample solvent should never be methanol when the modifier is ethanol or isopropanol. If the sample is dissolved in the same solvent as used as the modifier, the user should strongly consider switching the sample solvent to a much less strong (less polar)

solvent, particularly if the initial modifier concentration during injection is low (less than 10 % modifier). For analytical situations, this switch to a much less polar solvent is unlikely to create any solubility issues of the sample in the solvent. Most users underestimate the solubility of most smaller, drug-like molecules or larger natural-product type molecules in those less polar solvents.

The results in Figure 9.14, Figure 9.15 and Figure 9.16 were obtained with a 4.6 by 150 mm column with 5- μm particles. If the maximum injection volume, without band broadening, were found for the 4.6-mm id column, transferring the method to a column with smaller diameter or smaller particles, or both, the injection volumes should be decreased in direct proportion to the cross-sectional area of the column, and to the particle diameter. If the maximum injection on a 4.6-mm column with 5- μm particles is 10 μL , injecting onto a 3-mm id column with the same particles should be no more than about 4.2 μL . Simultaneously decreasing the particle size to 1.8 μm , while keeping the total efficiency the same (decreasing column length) requires an injection volume of no more than 1.5 μL . Obviously, with small inside diameter columns packed with sub-2- μm particles, a small loop may be required, which may not be readily available, commercially. A 1.25- μL loop can be made with a 10-cm length of 125- μm (0.005-inch) tubing. Using Agilent 1/32-inch outside diameter tubing with 1/16-inch ends makes this simple and easy to install. An alternative is the use of partial-loop injections, with a larger loop, but RSDs may not be adequate.

At higher modifier concentrations, this strong sample solvent effect is much less obvious. In fact, at 40 % methanol as modifier, injections of up to 90 μL of a warfarin standard dissolved in methanol could be injected onto a 4.6 by 250 mm column packed with Regispack stationary phase with only modest band broadening, for analytical scale semipreparative separations.

Before an injection, the wash pump in the Agilent analytical SFC system fills the loop with a wash solvent, chosen by the user. If this wash solvent is too strong a solvent, it needs to be displaced by several loop volumes of the actual sample dissolved in a weaker solvent (overfilling the fixed loop).

The worst-case scenario is to perform a partial-loop injection by inserting a small sample plug into the fixed loop, filled with a stronger solvent, but not displacing most of this stronger solvent with sample. A subsequent injection would then place a large volume of this highly polar (strong) solvent onto the column with the (weaker) sample plug, sweeping it down the column with most of the plug experiencing minimal retention, but

some of it having the strong solvent diluted down to where parts of the sample are far more retained. Subsequently, and in particular, the trailing edge is significantly broadened with a major tail.

If you are generating your own injection programs, an air bubble on either end of the sample plug creates *segmented flow*, which minimizes mixing between the sample and the wash solvent but can be used also to displace excess wash solvent. Large sample loops should be avoided. For method development, you should start with a small volume of a concentrated sample to try to minimize peak widths and maximize resolution, before trading resolution for sensitivity.

9.5 Miscellaneous considerations

9.5.1 Leaks

Liquid-leak detectors mandated by regulators for use in modern HPLC equipment are incapable of detecting or locating leaks in SFC, except, due to leaks in the degasser, or in the B-side of the binary pump since those modules are involved with pumping normal liquids. Elsewhere in the system, leaks will be gaseous with no dripping liquid. These types of leaks cannot trigger the liquid-leak detectors.

A visible leak, intermittently sputtering particles of dry ice, is a huge leak, generally meaning that the fitting in question is barely finger-tight. With such a leak, the software will probably shut down the system before the user has time to remedy the situation.

Much smaller leaks will be invisible to the naked eye, but may periodically freeze shut, then thaw, creating unstable pressure. This can cause either the booster pump, or the BPR, or both, to attempt to restabilize the pressure. The system may never get to the *ready* state, and will eventually shut down. Such leaks may make the fitting, where the leak is occurring, feel slightly cooler than the rest, but this not necessarily true. This should still be regarded as a major leak.

Built-in software-based leak tests, such as available for the Agilent analytical SFC control module can assist to detect leaks automatically, but this involves installing certain hardware items and running diagnostic software, which could take several hours. However, such functionality cannot detect the actual position of any leak.

There is a much simpler, faster, and more effective way to locate leaks based on vast experience in gas chromatography. A drop or two of soapy water applied to each fitting almost instantly indicates when there is a leak in that fitting, by making either a huge bubble, indicating a giant leak, or a few tiny bubbles, indicating an almost irrelevant tiny leak. There are commercial products designed to perform this task. Alternately, one

can dilute any household dish-washing detergent to perform the same task. However, some of the commercial products have a convenient, long 1/16-inch applicator tube that allows easy dispensing of a drop or two of soapy water onto each fitting.

Every time the chromatographic system is modified (for example, a change of column), every fitting that was loosened and refitted should be tested using soapy water immediately after pressurization, to determine whether even tiny leaks exist. Immediately after applying a drop or two of the solution, minuscule bubbles of less than to much less than 1-mm diameter may emerge from the fitting. However, if continuing observation of the fitting fails to find further bubbles, the fitting is tight. This should be the goal of every fitting in the entire system.

9.5.2 Ferrules

HPLC fittings were not necessarily designed to handle low viscosity fluids such as the mobile phases used in SFC. As a result, fittings are often overtightened to stop leaks. Two-piece, stainless-steel ferrules tend to deform after a relatively few number of disconnections and reconnections, and become progressively more difficult to seal. Eventually the ferrules can jam in the fitting. After deformation starts, it is recommended to replace the tubing.

Single-piece, stainless-steel ferrules tend not to distort on repeated retightening. However any slight bend in the tubing near the end can jam the ferrule in the fitting, making it close to impossible to remove the tube.

Polyimide ferrules tend not to distort and are easy to seal leak-tight, at least on stainless-steel tubes. However, they can jam in the fitting. Fortunately they can usually be removed easily, since they are relatively soft. PEEK ferrules are not recommended for use with stainless-steel tubing since such ferrules may not adequately hold the tube against the high pressures encountered and may fail, allowing the tube to be ejected from the fitting and vent the system. With PEEK tubing the author always uses stainless-steel ferrules, the tips of which can cut into the tubing, making a permanent geometry.

The most common location for repeatedly making, and remaking connections is in column end-fittings. Getting a fitting stuck in a column, often means you cannot get the fitting leak-tight, potentially ruining the column for further work. In the past, the author used a 10-cm piece of tubing connected to each end of a new column, with new tubing, nut and ferrules, and which was never removed from the column. This moved the vulnerable fitting out of the column and to the other end of the tube.

If that fitting fails, the tubing can almost always still be removed from the column and replaced.

Columns from different manufacturers can have different pilot-hole depths (the length of tubing that protrudes past the ferrule). Depths can change from 1.5 to at least 4 mm. This means that when changing columns, the connector tubing may also need to be changed, to avoid creating a mixing chamber or diffusion chamber, which would cause excessive band broadening.

Recently, Agilent has introduced several new fittings that largely solve these problems. In one type, the polyimide ferrule is attached to the nut. The nut can be screwed into the fitting until finger-tight. The tube is pushed through the ferrule until it touches the seat of the fitting. A lever can then be engaged, and locked in place, which presses the ferrule firmly against the seat with a spring of the appropriate tension to make a finger-tight seal. These seals are ideal for use with columns with different pilot-hole depths, since the depth is adjustable. These fittings are guaranteed for more than 200 make-and-break connections, and are recommended particularly for use in column end-fittings.

9.6 Ultrahigh performance with SFC

At the time of writing, there are no standard commercial SFC instruments configured as a true ultrahigh performance supercritical fluid chromatograph. In this context, ultrahigh performance SFC involves the use of sub-2- μm particles in columns with less than 4.6-mm id.

The mere use of such particles and columns does not indicate true performance. At a minimum, the instrument characteristics should be such that at least 90 % of the theoretical efficiency of the column should be achieved at k greater than or equal to 2.

SFC usually does not require an ultrahigh pressure instrument to use sub-2- μm particles, due to the much lower viscosity and resulting low-pressure drops of CO_2 -based mobile phases. Even at relatively high modifier concentrations such as 50 to 60 %, the pressure drop across a 100-mm column packed with 1.8- μm particles seldom exceeds 250 bar. However, the use of much longer columns or above optimum flow rates with smaller particles, makes a high-pressure system desirable. Although ultrahigh pump pressures are not necessary, other aspects of instrument design determine the capability to use sub-2- μm particles effectively

9.6.1

Flow cell volumes

Most chromatographs are designed with a specific column in mind, which dictates the size of all the components particularly that of the UV flow cell used. As a *rule of thumb*, any UV detector cell should have a volume less than one-fifth of the volume of mobile phase containing the fastest peak that the system is expected to produce. Larger cells cause extracolumn band broadening, and loss of efficiency and resolution between closely resolved peaks. For a perfectly packed 4.6 by 150 mm column, packed with 5- μm particles, the volume holding a peak with k equal to 1 is roughly (4σ) 150 μL . A flow cell of the order of 30 μL could be used. With a 4.6 by 150 mm column with 3.5- μm particles, the peak volume (k equal to 1) would be about 80 μL , requiring a cell volume of less than or equal to 18 μL , to avoid broadening the early eluting peaks.

The optimum flow rate for 1.8- μm particles in a 4.6-mm id column approaches 5 mL/min, which is the maximum flow for the Agilent analytical SFC system. Decreasing the column inside diameter to 3 mm decreases the optimum flow rate to about 1.7 to 2.0 mL/min, depending on the modifier concentration. With a smaller inside diameter, the cell volume must be significantly smaller. With a 3 by 100 mm column packed with 1.8- μm particles, the volume holding a peak with k equal to 1 would be about 15.5 μL and the cell volume would need to be less than 3 μL . With a 2.1 by 100 mm column packed with 1.7- μm particles, the peak volume with k equal to 1 would be theoretically 7.3 μL , requiring a cell volume less than 1.5 μL .

Recently, a new 3 mm, 2- μL flow cell has become available that has a conical through-hole, and is much less susceptible to thermally induced noise. This type of flow cell is highly recommended when using columns packed with sub-2- μm particles.

9.6.2

Tubing

The tubing connecting the injection valve to the column, and the column to the UV detector can also contribute significantly to extracolumn band broadening. None of the currently available SFC systems are designed to have tubing diameter or length to make them truly ultrahigh performance SFC systems.

The variance of a peak caused by the dispersion of the connecting tubing is directly proportional to tubing length, and to the fourth power of the radius. The standard 175- μm id tubing in the Agilent analytical SFC system has a too large inside diameter and is too long for use with sub-2- μm particle packed columns. Therefore, instrumentation to be used with sub-2- μm particles needs to be slightly modified in order to achieve optimum performance.

With the smaller 3-mm detector cell and most of the tubing replaced with 125- μm id tubing, the Agilent analytical SFC system can deliver approximately 90 % of the efficiency of a 3 by 100 mm column packed with 1.8- μm particles, and with k as low as 2.5. This type of column can produce about 20 % higher efficiency in less than one-third of the time, compared to a 4.6 by 150 mm column packed with 3.5- μm particles.

9.7 Hybrid systems switching between SFC and HPLC

The Agilent analytical SFC/UHPLC hybrid system can be configured as a hybrid system¹¹⁷ that can be switched rapidly back and forth between SFC and either normal phase or reversed phase HPLC operation, even within a sequence. This is surprising since water is miscible with CO_2 at no more than a few tenths of 1 %. However, once a polar modifier is present, it is much easier to flush the system between techniques. An additional pump, typically a quaternary pump, is configured along with a 2-position/10-port valve. The regular SFC binary pump is only used in SFC mode. The additional pump is used only to deliver the HPLC solvent mixture, which is most often aqueous-based. The two systems share the degasser, autosampler, column compartment and detector. A schematic diagram is presented in Figure 9.17 for the system operating in SFC mode. Figure 9.18 presents the system operating in HPLC mode. Both a reversed phase and a polar column are mounted in a single oven on a 2-position/6-port column-selection valve or using a method development setup that facilitates automated screening of multiple columns¹¹⁷.

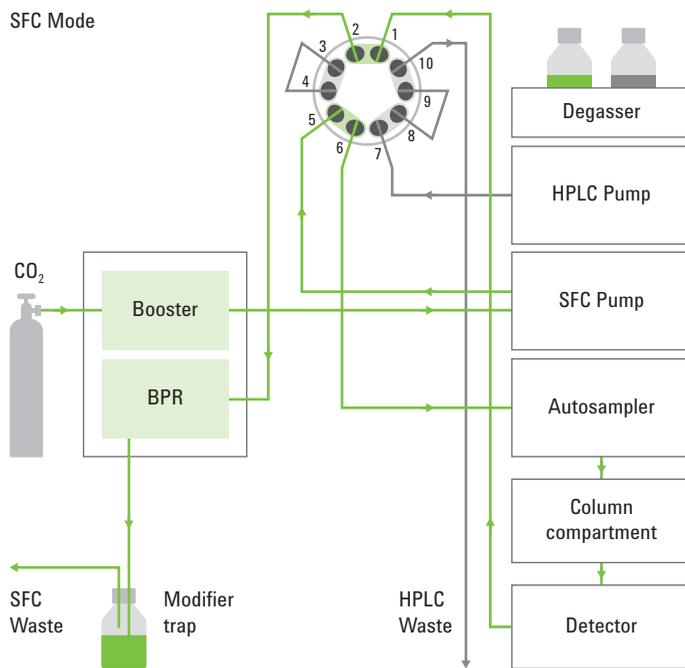


Figure 9.17 Schematic of the Agilent analytical SFC/UHPLC hybrid system configured for SFC operation.

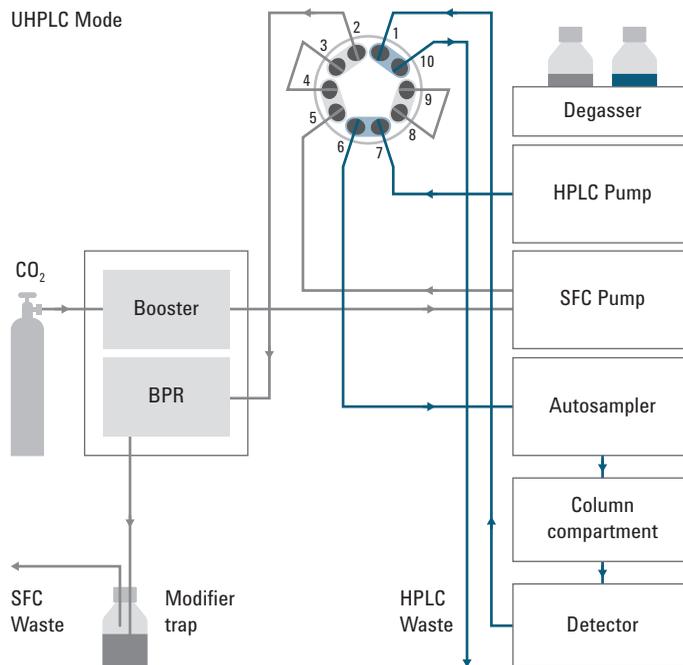


Figure 9.18 Schematic of the Agilent analytical SFC/UHPLC hybrid system configured for UHPLC operation.

When the valve is switched to change techniques, a small amount of the mobile phase from the *other* technique may enter either column, but is quickly re-equilibrated. Switchover takes no more than a few minutes.

A mix of eight compounds from three different solute families; caffeine, theophylline, cortisone, prednisone, hydroxycortisone, prednisilone, sulfamerazine, and sulfaquinolaline were separated¹¹⁸ by both SFC and UHPLC on the same equipment. Six SFC runs were followed by two washing runs, and a blank to clear the system of the SFC mobile phase. Six UHPLC injections were performed, followed by one SFC wash and a blank. Four more SFC runs were performed, followed by two washes and a blank, and four UHPLC runs, further washes, and finally, two extra SFC runs. All the runs were controlled as part of a single automated sequence. All 12 of the SFC runs were overlaid and are presented in the top of Figure 9.19. The authors reported that the retention-time RSD for isocratic separations was between 0.04 and 0.08 %, while gradient runs produced an RSD between 0.02 and 0.04 %.

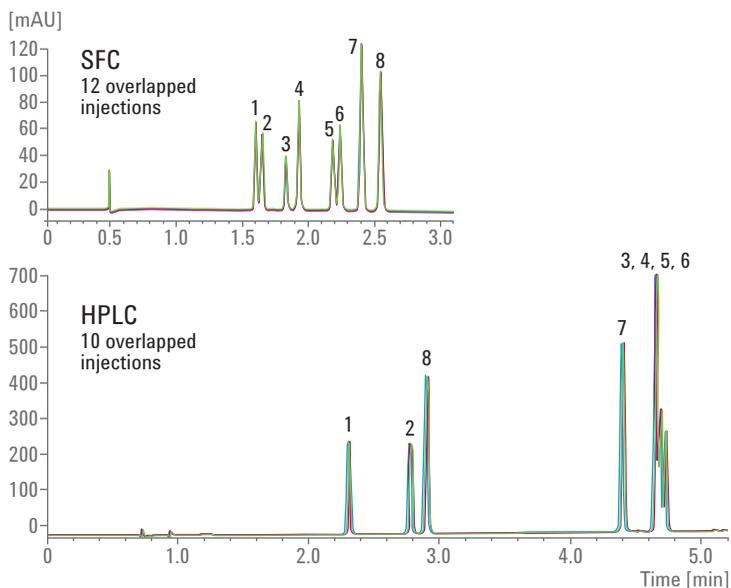


Figure 9.19 Results of a sequence where six SFC runs, followed by six UHPLC runs, four SFC runs, four UHPLC runs, and finally two SFC runs were made. The system was flushed with several gradient washing blanks during switchover between methods. All 12 SFC runs were overlaid, as seen in the upper chromatograms. Similarly, the reversed phase HPLC runs were all overlaid as shown in the lower chromatograms.

The 10 UHPLC chromatograms were also overlaid (lower panel in Figure 9.19), showing retention-time reproducibility with RSDs about 0.2 %. Comparing the results from the two methods demonstrates once again the orthogonality of the two techniques, since the elution order is significantly different in the two data sets. Such large differences in selectivity should be welcome in situations where two orthogonal methods are required. For example, to ensure that trace contaminants are not hiding under a major peak. In this particular comparison, the UHPLC method uses a 2.7- μm packing compared to the 5- μm packing used for SFC.

9.8 Interfacing to mass spectrometers

Starting more than 25 years ago, almost every kind of mass spectrometer has been interfaced to SFC systems. As mass spectrometer designs have improved, the interfaces have become simpler. In fact, it is now feasible to directly couple the outlet of the BPR to the inlet of the MS source, simply using a piece of 125 to 250 μm stainless-steel tubing, although this tends to broaden peaks somewhat, see Figure 9.20. Most of the pressure drop will occur across the BPR but there will be substantial back pressure caused by the tubing. Nevertheless, by the time the fluid reaches the source, most of the mobile phase will have vaporized, becoming a poor solvent. A make-up pump delivering a small flow of modifier, usually methanol, is used to ensure none of the solutes stick to the walls of the transfer line. There are no issues with changing split ratios when the composition changes.

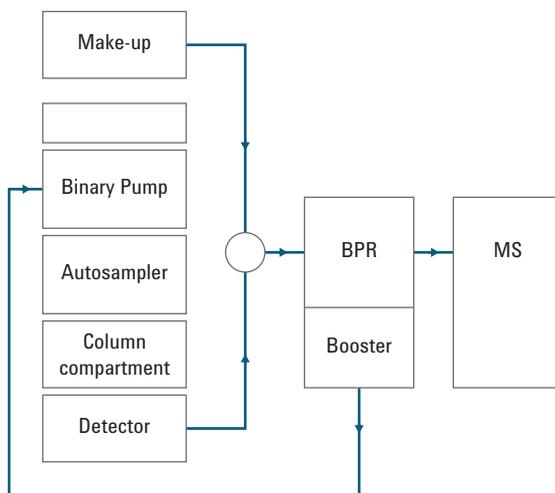


Figure 9.20 Straightforward interfacing of SFC to MS. The total flow passing through the BPR is fed into the MS source. A make-up pump adds a small flow of a polar modifier and additive before the BPR.

An alternate interface, consisting of several T-pieces and a 1-meter length of 50- μm id stainless-steel tubing is shown schematically in Figure 9.21. After the UV detector, the flow is split with a T-piece. Typical split ratios are 5:1, with the lower flow going to the MS, while most of the flow passes through the BPR. It is useful to have a make-up pump for this configuration also. The make-up flow is added either before, or after the split, through another T-piece. The restrictor is mounted downstream of this second T-piece, and directly onto any APCI or ESI source. The make-up flow insures there is enough liquid entering the MS for proper ionization, and may be used to minimize changes in split ratio caused by changes in composition and viscosity.

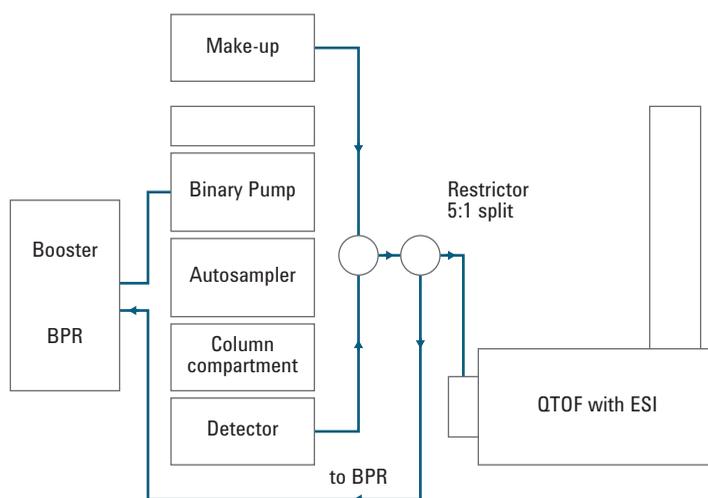


Figure 9.21 Alternate interfacing scheme where two T-pieces after the detector allow the addition of modifier from a make-up pump, and allow the flow to be split. In this case, 1 meter of 50- μm stainless-steel tubing was used to create a split ratio of about 1:5 with the lesser amount going to the MS.

The hybrid system is most versatile when used with a mass spectrometer as an additional detector. The ability to switch rapidly back and forth between SFC/MS and HPLC/MS is a powerful capability, particularly for trace analysis where orthogonal methods are needed. The combination of a *normal phase* SFC with reversed phase HPLC, should be viewed as greatly superior to two reversed phase HPLC methods,

9.9 Other Detectors

The same kind of split-flow interfacing used with mass spectrometers is appropriate for a number of detectors mostly used for HPLC. These detectors operate at atmospheric pressure.

9.9.1 Evaporative light scattering detectors

The most common detector used in HPLC that operates at atmospheric pressure is based on evaporative light scattering (ELS)^{119–121}. Evaporative light scattering detectors have been interfaced to SFC systems for more than 25 years. An ELS detector is a nearly universal, so that the samples do not need to have a UV chromophore. The same split is appropriate as described in Section 9.8 “Interfacing to mass spectrometers”.

9.9.2 Gas chromatography detectors

In the past, SFC systems often deployed a gas chromatograph as the column oven. This made it easy to interface most GC detectors. Most GC detectors have been interfaced to packed column SFC. They include the flame ionization detector (FID) electron capture detector (ECD), nitrogen phosphorous detector (NPD), and sulfur chemiluminescence detector (SCD).

Most GC detectors were used with a large split ratio with only a small fraction of the total flow entering the detector (depending on the type) while the rest was vented through the BPR. These detectors were used mostly with 100 % CO₂, which requires a different form of restrictor compared to those discussed so far. The restrictors consisted of a 25 to 30-cm piece of 50- μ m id fused silica tubing with the tip drawn down to a few μ m over the last 1 to 2 mm of length. The pressure drop occurred almost exclusively over this last short section, keeping solutes in solution to the last moment. Larger versions of such restrictors can be used with mass spectrometers and ELS detectors but have a tendency to plug and are more difficult to work with.

9.9.2.1 Flame ionization detectors

Flame ionization detection was widely used with both capillary and packed column SFC, primarily with pure CO₂ as the mobile phase, although a few other pure fluids were used. In a few cases, small concentrations of water were added to the CO₂ to enable the elution of more polar solutes like free fatty acids.

There are several ASTM SFC methods that are still widely used for the analysis of aromatics in diesel, and olefins in gasoline. Both use pure CO₂ and an FID. Unlike most of the older methods that use pressure or density programming at much higher temperature, both these petroleum applications use a constant outlet pressure and temperatures near room temperature, which makes them compatible with the Agilent analytical

SFC system. Recently, a standalone FID based on Agilent hardware was introduced, which can be interfaced to Agilent chromatography data systems.

The FID can tolerate only relatively low flows of CO₂. The petroleum methods have traditionally been performed on either a 4.6-mm id column or on micropacked columns. By far the fastest, highest resolution, most reproducible results are obtained with the larger columns, not the micropacked columns. With 4.6-mm (or 3 or even 2.1-mm) columns, a relatively large split ratio is required, partly because the samples are neat. The design of the restrictor is a key element. There can be no make-up flow since the FID is a near *universal* detector and has a large baseline offset when a modifier is present. The CO₂ gas flow rate (at atmospheric pressure) into the FID is usually around 10 to 40 mL/min. The flow through a 4.6-mm id column is on the order of 1000 mL/min when measured at atmospheric pressure. Thus, the split ratio needs to be 25:1 to 100:1. This requires an *integral* restrictor (a pinhole with a diameter of 1 to 2 μm) or a linear restrictor of less than 25 μm of significant length. This is similar to the requirements for most other GC detectors such as a nitrogen phosphorous detector, the electron capture detector, and many others although many of these can be used with modified fluids.

9.9.3 Chiral detectors

Several chiral detectors, capable of differentiating the right- and left-handedness of enantiomers, which have been widely used in HPLC, have been adapted for SFC. Both circular dichroism detectors and laser polarimeters have been available. These detectors are capable of handling the full flow through the column at high pressure. There is no splitter required. Such detectors allow you to determine the elution order of the enantiomers. Screening with various columns usually involves racemic mixtures, which makes it easier to determine which enantiomer elutes first. Ideally, when doing enantiomeric excess determinations, the much lower concentration enantiomer should elute first, avoiding any tailing of the higher concentration enantiomer, thereby enabling more accurate quantification. However, without a chiral detector, there is no easy way to rapidly determine which enantiomer elutes first without collecting peaks and analyzing offline.

9.9.4

Missing detectors

The most obvious missing detector is a fluorescence detector. All the existing commercial HPLC fluorescence detectors are incompatible with the high outlet pressures needed. The mechanical layout of existing detectors makes it unlikely that a high-pressure cell could be fabricated.

One of the most widely used detectors in HPLC is the refractive index (RI) detector. From previous discussions, it should be obvious that the large changes in RI occurring during a gradient of CO₂ with any of the common modifiers rules out its use.

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