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Review

Separation of polar solutes by packed column supercritical fluid chromatography

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Abstract

The application of packed column supercritical fluid chromatography (SFC) for the separation of polar analytes is reviewed. A comparison is drawn between the separation of polar and non-polar analytes on open-tubular column SFC. The different column materials used in packed column SFC are considered. The need for modifiers and additives to the carbon dioxide eluent for packed column separations is discussion and the influence of the instrumentation on the development of the method is described. The application of packed column SFC to a wide range of structure types of acidic, neutral and basic analytes and of chiral analytes is reviewed and the effect of the conditions are compared. © 1997 Elsevier Science B.V.

Keywords: Reviews; Columns; Packed columns; Supercritical fluid chromatograph; Stationary phases, SFC; Mobile phase composition; Acids; Basic compounds; Polar compounds

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1. Introduction

This review covers the separation of "polar" solutes using supercritical fluid chromatography (SFC). The word "polar" is used to describe solutes that do not elute or elute with poor peak shapes from packed columns, using pure carbon dioxide as the mobile phase. Roughly, the solute polarity range of interest in this review begins with phenol and aniline, as the least polar, and ends with polyfunctional aliphatic amines and polyfunctional acids at the most polar extreme.

The emphasis on mobile phase selection centres on binary and ternary mixtures of carbon dioxide with organic solvents and additives. Since such fluids are rarely used with capillary or open tubular columns most references to polar solutes involve packed column separations.

No differentiation will be made between what is sometimes called subcritical fluid chromatography (subSFC, or sSFC), near critical fluid chromatography, enhanced fluidity chromatography and SFC. "Transitions" between these "defined" states are undetectable chromatographically and the instrumentation used is identical.

The material is presented from several perspectives. The first two sections approach the separation of polar solutes in terms of mobile phase and stationary phase effects. A short section then discusses the role of instrumentation in the extension of

SFC to more polar solutes. A number of sections deal with separations of specific functional groups. Chiral separations are discussed in a separate section. A final section gathers up miscellaneous materials.

This review is not exhaustive. When there are many publications on a specific topic representative papers are discussed.

2. The mobile phase

2.1. Pure fluids

Most of the earliest work in SFC involved the analysis of relatively non-polar polymers. One of the earliest attempts (1969) to extend SFC to more polar solutes was by Sie et al. [1]. They used moderately polar fluids like pure isopropanol, at high temperatures with non-silica based, microporous polymer beads as the stationary phase. Acetylsalicylic acid, caffeine, mono-, di- and tri-hydroxyphenols, and other solutes of interest in pharmacology were eluted and separated.

In 1968, Giddings et al. [2] over-estimated the Hildebrand solubility parameter for carbon dioxide, placing it beside isopropanol in solvent strength. If true, pure carbon dioxide would be surprisingly polar and should dissolve relatively polar solutes, like those eluted by Sie et al. [1], but at much lower temperatures. We now know that carbon dioxide is much less polar than this early estimate. Carbon dioxide is similar to pentane, not isopropanol. Nevertheless, it is still widely believed that carbon dioxide is much more polar than it actually is. For a multitude of reasons, carbon dioxide has become the fluid of choice for SFC.

2.1.1. Solubility in carbon dioxide

Solutes must have appreciable solubility in a fluid for that fluid to be an effective chromatographic mobile phase. Many substances with single polar functional groups are surprisingly soluble in carbon dioxide. However, more complex, polyfunctional solutes tend to be much less soluble.

Formic and acetic acids are miscible [3,4]. Both phenol and aniline are soluble [3,4] to 3% (w/w) in liquid carbon dioxide at 25°C. Nevertheless, each is sometimes difficult to elute from either capillary or packed columns with pure carbon dioxide. Less soluble solutes are less likely to be eluted with carbon dioxide.

Stahl [5] reported that hydrocarbons and other lipophilic materials, like ester, ethers, lactones and oxides are soluble in pure carbon dioxide. However, he also stated that the addition of polar functionality, specifically hydroxyl or carboxyl, decreased the extractability of a substance. Further, very polar substances like sugars (polyfunctional) and amino acids (zwitterions) were not appreciably extracted using carbon dioxide at up to 500 bar.

Dobbs et al. [6] reported that the solubility of benzoic acid varied from 0.1 to 0.3 mol% with pure carbon dioxide at 35°C between 0.75 and 0.97 g/cm^3. The solubility of 2-aminobenzoic acid varied from 0.008 to 0.013 mol% under the same conditions. Solubility of each increased at least a factor of 5 when 3.5 mol% methanol was added to the mobile phase.

The mole fractions of cholesterol, stigmasterol and ergosterol in pure carbon dioxide at 35°C were much lower, of the order of $5 \cdot 10^{-5}$ ($5 \cdot 10^{-3}$ mol%), 10^{-5} and 10^{-6} over the same density range [7]. Ashraf-Khorassani et al. [8] reported that several moderately polar sulfa drugs were only weaker soluble at approximately 11 ppm in carbon dioxide. In 1978, Stahl et al. [9] reported the solubility of several amino acids and sugars at extreme pressures. With carbon dioxide at 40°C and 2000 bar, solubilities were: glycine, 0.40 μg/l; L-leucine, 0.55 μg/l; sucrose, 1.5 μg/l; glucose 2 μg/l and xylose, 3.0 μg/l. Most of these very low solubility measurements have been available for more than a decade suggesting that successful chromatography with carbon dioxide alone would be unlikely. Despite such knowledge, there have been many attempts to separate many of these polar solutes using pure carbon dioxide.

2.1.2. Other pure fluids

Carbon dioxide remains the fluid of choice for SFC, due to its modest critical point, low cost, available purity, safety, ease of use and lack of a viable alternative. Many other fluids have been tried, without discovering an alternative. Giddings et al. [2] suggested the use of supercritical ammonia for the

separation of polar molecules, including biopolymers. However, results with ammonia have been irreproducible (possibly due to variable water content), and the potential dangers associated with its use mean that few are willing to work with it routinely. Lauer et al. [10] showed that pure ammonia was effective in eluting moderately polar bases. Ammonia was also recently used as a chromatographic mobile phase [11] in capillary SFC for the separation of polar drugs, amino acids and herbicides.

In efforts to extend the polarity range, many other pure fluids have been tried. Sulfur dioxide is polar but too corrosive [12] and is difficult to obtain in pure form. Nitrous oxide has been used for numerous SFC studies but exhibits solvent strength similar to carbon dioxide. Since it is an extreme oxidising agent, it should not be mixed with organic solvents (i.e., fuels). At least one death has resulted from a nitrous oxide/fuel explosion associated with SF separations.

Numerous chlorofluorocarbons and, now fluorocarbons, have been used as mobile phases for SFC (i.e., [13–17]). Some have high dipole moments and, in certain instances, appear to be much more polar than carbon dioxide, although they are not appreciably acidic or basic or participate in hydrogen bonding.

The hydrofluorocarbons such as F-23 and, especially, F-134a [13,14] have shown promise as chromatographic mobile phases. Since F-134a has become a common refrigerant, its price has dropped dramatically, which is likely to result in its more widespread use.

Having tried many other fluids in efforts to extend the polarity range, the general lack of success, or danger, has caused most later workers to avoided high temperatures, flammable/explosive or highly corrosive fluids and return to carbon dioxide with added polar organic modifiers.

2.2. Binary mobile phases

Throughout the 1980's, most interest in SFC revolved around capillary methods, particularly in the USA. However, many groups continued to explore the use of polar modifiers (and, typically, packed columns) to separate polar solutes [18–23].

2.2.1. Alcohols in hydrocarbon eluents

Before 1980, short chain alcohols were often used as modifiers in pentane or another short chain hydrocarbon. These mixtures have high critical temperatures (usually >200°C). Using such mixtures near their critical temperature can be dangerous and negates their use for the analysis of thermally labile solutes. With 95% pentane, 5% methanol, no resolution of styrene oligomers was observed until the temperature was >180°C [24,25]. With 5% isopropanol in *n*-pentane, resolution was lost near the critical point (of *n*-pentane) when a negative temperature program was used.

2.2.2. Modifiers for carbon dioxide compatible with the flame ionization detection

One of the major advantages of capillary SFC over high-performance liquid chromatography (HPLC) is its compatibility with flame ionization detection (FID). FID provides universal, sensitive detection of carbon compounds, with a uniform response factor, while allowing pressure or density programming. Unfortunately, FID is incompatible with most organic modifiers, yet modifiers were generally required to dissolve polar solutes. A search for polar modifiers that did not respond to FID led to the use of water or formic acid.

2.2.2.1. Water

A British patent [26] first reported the use of either pure or wet carbon dioxide for the extraction of caffeine from tea leaves. However, water is only sparingly soluble in carbon dioxide. Consequently, the various techniques for saturating the mobile phase with water, developed for normal-phase HPLC, have been applied to SFC.

Geiser et al. [27] reported the separation of underivatized fatty acids using water in carbon dioxide, with FID. Engelhardt et al. [28] showed that the addition of water to carbon dioxide significantly improved the peak shapes of both weak acids and bases (i.e., 2,6-dimethylaniline) on silica based columns. France et al. [29] used a Deltabond Octyl column with water saturated carbon dioxide for the analysis of fatty acids, di- and triglycerides in abused vegetable oils. Only a few groups [30–32] have continued developments along these lines.

2.2.2.2. Formic acid

Formic acid also does not respond in FID. It has been used as a modifier in capillary SFC [21,33]. Schwartz [34] separated a group of mycolic acids using 0.3% formic acid, 0.17% water in carbon dioxide at 100°C, and a micropacked column. Hanson et al. [35] found that the addition of formic acid, to methanol, narrowed peaks of α, and β unsaturated aldehydes and related compounds on packed columns.

2.2.3. Organic modifiers in carbon dioxide

Although apparently not reported until 1982 [36], the most common modifier–main fluid combination used today is methanol–carbon dioxide. Small concentrations of polar modifiers dramatically increase the solvent strength of the mixtures. The enhanced solvent strength of the fluids is caused by their composition, not by any increase in density associated with the addition of the modifier [37]. Solvatochromic dyes have shown that small additions of methanol to carbon dioxide produce a disproportionate increase in solvent strength. For example, the addition of 5% methanol to carbon dioxide shifts the solvent strength as much as one would expect from 20% methanol.

Polar modifier molecules tend to cluster together, forming localized regions of high polarity in the bulk mobile phase [38,39]. The non-linear solvent strength observed with solvatochromic dyes correlates with the non-linear retention observed when retention is plotted as a function of modifier concentration. At low temperatures (i.e., 40–60°C), methanol–carbon dioxide mixtures are unstable [40] below approximately 0.5 or 0.6 g/cm^3 (below 80–100 bar). Thus, there is a smaller range of densities available for adjusting retention with binary fluids, compared to pure fluids. Mixtures of low concentrations of methanol in carbon dioxide have virtually the same physical characteristics (viscosity and diffusivity) [41] as carbon dioxide.

Randall (1984) [18,42], Blilie and Greibrokk (1985) [21], and Levy and Ritchey (1986) [20] all published relatively early systematic studies of the effect of modifiers on the retention of polar solutes using modified carbon dioxide and packed columns. Randall's first choice for a binary solvent pair was methoxyethanol in carbon dioxide. Methoxyethanol is slightly more polar than methanol on Snyders P' scale (5.5 vs. 5.1). There have also been attempts (i.e., [43]) to predict retention based on solvent properties.

2.2.4. Solubility in binary fluids

The solubility of cholesterol, stigmasterol and ergosterol increased as much as an order of magnitude when modifiers were added to carbon dioxide [7]. However, perhaps surprisingly, acetone, as modifier, increased solubility much more than ethanol, which increased solubility more than methanol. Methanol modified carbon dioxide produced results almost the same as pure carbon dioxide. The apparent ineffectiveness of methanol in improving the solubility of these low polarity solutes mirrors chromatographic experience in the mid-1980's. Retention of modestly polar polycyclic aromatic hydrocarbons (PAHs) was nearly unchanged when pure and methanol modified carbon dioxide were compared. This was widely interpreted as showing that polar modifiers did not change the solvent strength of carbon dioxide. This was further interpreted as indicating that carbon dioxide was as polar as alcohols (as per Giddings erroneous estimate of solvent strength). What little change in retention occurred was attributed to changes in the density of the fluid.

During this time period, no polar solutes were used as probe molecules. However, if more polar probe molecules are used, the effect on adding a polar modifier is different and for very polar solutes, can cause large increases in solubility and large decreases in retention. For example, in 1987, Dobbs et al. [6] reported that the solubility of benzoic acid and 2-aminobenzoic acid each increased by more than a factor of 5 on the addition of 3.5% methanol.

2.3. Ternary mobile phases

The first use of relatively small amount of further components, such as traces of acids and bases, as additives to the SFC eluent [44] appeared in 1988. Although it was originally thought that the mechanism for improving peak shapes was ion pairing [44–46], it was later found [15,47] that, in most cases the mechanism appeared to involve suppres-

sion of ionization by the solutes, with a few examples of ion pairing [46].

Most strong organic bases will not elute or eluted with extremely poor peak shapes from packed columns using pure carbon dioxide (Fig. 1A), pure Freon-23, or pure Freon-13. The addition of methanol caused the solutes to elute but with very poor peak shapes (Fig. 1B) [15]. The addition of a basic additive to the mobile phase caused a dramatic improvement in peak shapes (Fig. 1C).

Most polyfunctional organic acids and hydroxy acids will not elute or elute with poor peak shapes using packed columns and methanol modified carbon dioxide. There have been a number of papers studying the effectiveness of various additives in improving the peak shapes of acidic solutes [47–50]. In general, stronger acids make the best additives for suppressing tailing and improving peak shapes of acidic solutes.

3. The stationary phase

3.1. Phase ratio differences in packed and capillary columns

The most confusing single aspect of retention in SFC involves comparing retentions on capillary and packed columns. Retention on any column type is directly proportional to the phase ratio (β) [β=(void volume)/(stationary phase volume)] of a column [51]. Typical packed columns are 10- to 100-times more retentive than typical capillaries, due solely to differences in the "phase ratio" [52]. Using the same fluids for both capillary and packed columns will therefore produce very different retention characteristics. Part of the reason packed columns often require modified mobile phases is this greater inherent retention capacity.

During the mid-1980's, considerable effort was expended (incorrectly) trying to show that polar organic modifiers, did not significantly extend the polarity of carbon dioxide [53–56], and implied that the greater retention on packed columns was due to the active (reactive, poor quality, inferior, etc] nature of the packing (rather than differences in phase ratio). This helped fuel the unfortunate controversy over whether packed or capillary columns were "superior". This controversy diverted attention from the fact that the two techniques tend to best address different problems.

3.2. Column activity

In efforts to retain the use of pure carbon dioxide, there have been many attempts to either deactivate silica based packing materials or use polymer based packings. These efforts continue.

De Weert et al. [57] used phenol as a probe of silanol activity on numerous silica based stationary phases, including endcapped and polymer deactivated versions, using pure carbon dioxide as the mobile phase. On Deltabond cross-linked cyano columns [58] both phenol and 2,6-dimethylaniline showed significant improvement in peak shapes compared to a similar but non-deactivated column, using pure carbon dioxide as the mobile phase. Caffeine gave sharp peaks using pure carbon dioxide and pressure programming. Several other weak bases were also separated with acceptable peak shapes. The authors were able to elute several tertiary aliphatic amines with pure carbon dioxide, but at relatively high temperature and with relatively severe tailing.

Both anilines and benzylamines gave poor peak shapes [16] on Deltabond columns using pure carbon dioxide. The addition of methanol improved the peak shapes of the anilines but results were no better than on an undeactivated column with a similar stationary phase. Ternary mobile phases improved the peak shapes of benzylamines [15,59] on the Deltabond deactivated column but no better than on an undeactivated column.

Shen and coworkers [60–62] continue to develop advanced deactivation schemes to suppress such activity while also producing relatively polar stationary phases. The approach has been surprisingly successful, resulting in the elution of strong bases [60], including some primary aliphatic amines using only pure carbon dioxide (without additive or even modifier) as the mobile phase. More recent, as yet unpublished results [63] show even more promise for the separation of strong bases using pure carbon dioxide and a microcolumn packed with a diol bonded phase coated with PEI (a polyamine with 750 000 av. molecular mass).

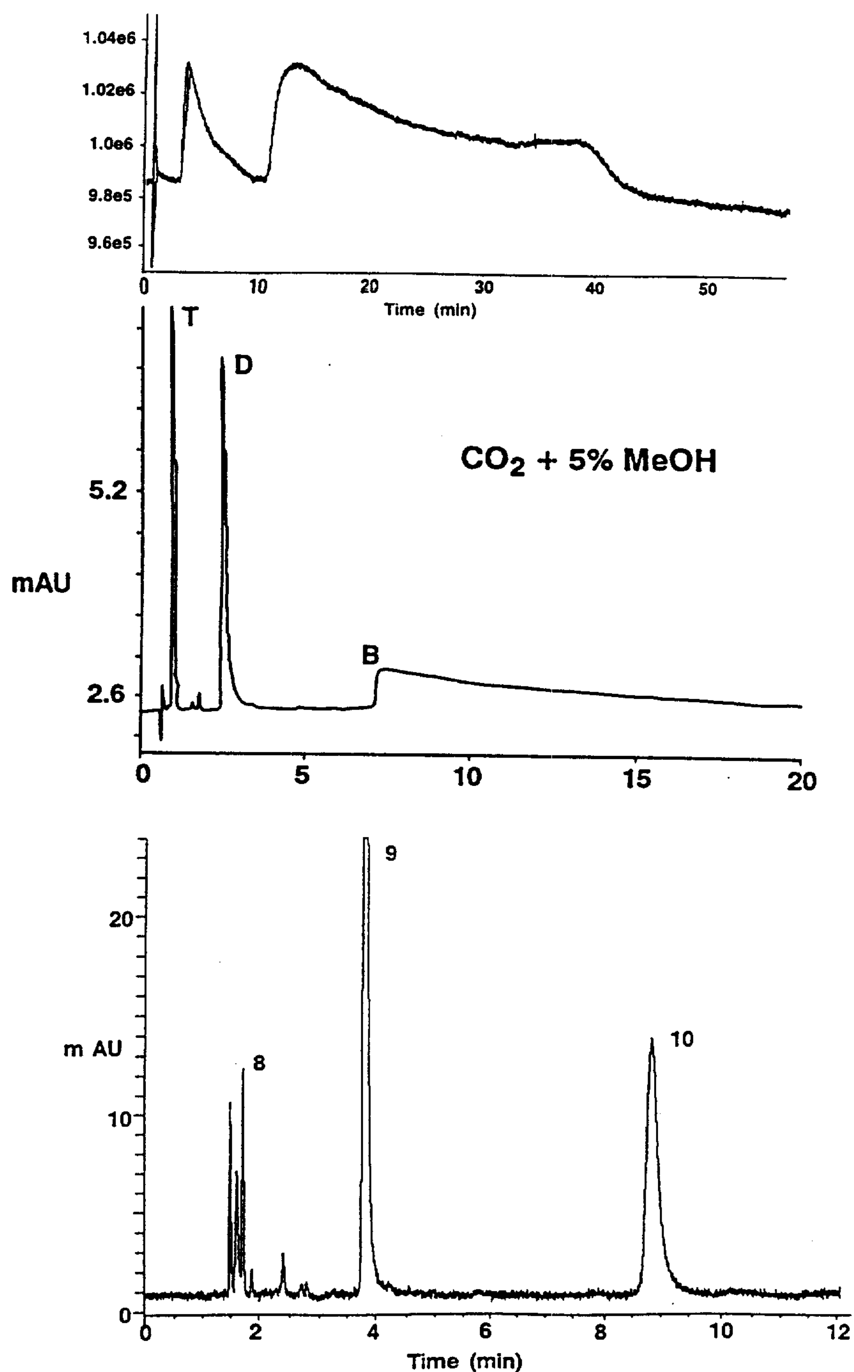

Fig. 1. (A) Benzylamine eluted from a Deltabond Octyl column using pure carbon dioxide as the mobile phase. 100×2 mm, 5 μm, 0.5 ml/min, 40°C, 182 bar. (B) Separation of three benzylamines, including benzylamine (B) on a Diol column, using 5% methanol in carbon dioxide; Nucleosil Diol 100×2 mm, 7 μm, 0.5 ml/min, 40°C, 182 bar. (C) Separation of same three benzylamines, including benzylamine (10) using a Diol Column and a ternary mixture of 10% methanol (containing 0.6% isopropylamine) in carbon dioxide; 250×4.6 mm, 5 μm Lichrospher Diol, 2 ml/min, 40°C, 200 bar.

Polymer based packing materials [64–67] are periodically used to avoid the "activity" of silica, to try to elute more polar acids using pure carbon dioxide. Yang [64,65] separated acids on a polystyrene–divinylbenzene column. Peak shapes similar to those obtained with silica based columns resulted. Gere used PRP-1 polymer based stationary phase to elute some weakly basic solutes, but with modifiers. Lauer et al. [10] also used PRP-1 columns when using ammonia as the mobile phase to elute caffeine, theophyline and nicotine. Smith and Sanagi [66] demonstrated elution, but poor peak shapes, for both benzoic acid and benzamide with pure carbon dioxide from a polymer based polystyrene–divinylbenzene packed column. Small concentrations of methanol dramatically improved peak shapes.

A column containing polymer based particles allowed the elution [59] of a test mix containing anilines, benzylamines and stronger aliphatic amines, using methanol–carbon dioxide as the mobile phase (without an additive). However, the peaks exhibited low efficiency and tailed badly. The overall quality of the separation was poor compared to either separations of the same mix on a silica based column using a methanol–carbon dioxide mobile phase with a basic additive [43], or Shen and Lee's PEI coated diol column [63].

France et al. [40] suggested that additives decrease tailing by interacting with, or blocking access to active sites on the packing and that a better deactivated column would produce similar results without the additive in the mobile phase. Corticosteroids [67] tail on C_8 but give good peak shapes on more polar phases using methanol modified carbon dioxide. Additives were thought to suppress unwanted interactions with extraneous, polar "active sites" on the less polar stationary phase. However, additives, even trifluoroacetic acid, did not improve the peak shapes on the less polar column, yet were unnecessary on the more polar columns. These results indicate that the traditional explanation of tailing, based on interactions of polar solutes with active sites, is completely inadequate.

3.3. Modified fluids change the stationary phase

Mobile phase components are strongly adsorbed onto the stationary phase and can change both its volume and polarity [49,68–70]. Adsorption is most extensive at temperatures <40 or 50°C from the critical temperature. Additives adsorb onto many, but not all, stationary phases. Surface coverages between 0.4 and 21% were measured from millimolar, or lower, mobile phase concentrations. The amount adsorbed increased with increasing stationary phase polarity. Changing the concentration of the modifier appears to affect the amount of additive adsorbed. The higher the concentration of modifier the smaller the amount of additive adsorbed. Without changing the concentration of the modifier but changing the concentration of the additive, surface coverage was essentially constant.

Perhaps surprisingly, very little effort has been expended to try to combine capillary and packed column SFC by decreasing the retentivity of packing materials through decreasing surface area (i.e., deactivated, non-porous silica), and decreasing pressure drops (i.e., using longer beds of larger particles).

4. Instrumentation

Over several decades, inadequate instrumentation caused a number of misinterpretations and false directions in the development of SFC. For example, up until the late 1980's, it was generally believed that the addition of modifiers did not increase the solvent strength of carbon dioxide. Changes in retention were attributed to changes in the density of the fluid. It is now known [37] that polar modifiers dramatically change the retention of polar solutes, and that changes in the density of binary fluids cause smaller shifts in retention.

4.1. Pumps

During the 1980's, most SFC systems used syringe pumps, operated as pressure sources. Flow was passively controlled with a fixed restrictor mounted on the end of the column. The main problem with syringe pumps is the inability (or at least great difficulty) in controlling individual parameters such as flow-rate, density, temperature and composition, independent of each other, and determining the effect of any one variable on retention. Such systems are ideal for controlling pressure, but not flow. A change

in pressure results in an ill-defined change in flow [71]. If the pressure is changed, the retention time of solutes will change. Both the density of the fluid and the amount of fluid flowing change with pressure. Consequently, the effect of density on retention is intricately linked with the effect of changes in the flow-rate on retention.

Using multiple syringe pumps to control composition is difficult, if not impossible, since one of the fluids is compressible (even when chilled), while the other is relatively incompressible. To generate precise compositions, the pumps must be used as flow sources, with an independent device controlling pressure. When the pressure is changed, part of the piston displacement in the pump delivering the compressible fluid goes toward compressing the fluid, while part of the displacement delivers flow to the column. Either the motor driving the piston must change speed to compensate for the compressibility change, or the flow of that fluid will change. The amount of piston displacement required to only compress the fluid depends on the volume of fluid remaining in the piston. Since the other fluid (in the other pump) is incompressible, its flow remains constant. If the compressible fluid flow changes while the incompressible fluid remains constant, an uncontrolled composition program results.

To allow use of syringe pumps with binary fluids, premixed binary mixtures were developed by gas supply companies. A single syringe pump was operated as a pressure source, with a fixed restrictor to limit flow. The flow through a fixed restrictor also depends on the fluid composition. Thus, the effects of pressure, flow and composition on retention are all confused. It becomes nearly impossible to plot the effect of one variable on retention while, holding all the other variables constant.

It is now known [72,73] that the composition of the fluid withdrawn from premixed cylinders changes as the cylinder is used up. The methanol–carbon dioxide composition delivered from the cylinder changes by a factor of two from the first to last use of a pre-mixed cylinder. As retention is approximately inversely proportional to methanol concentration, doubling the concentration halves retention.

The recognition that with binary fluids, composition was more important than density is a relatively recent discovery. Before this recognition, workers attempted to work at high temperature with binary fluids of constant composition, and control retention by programming pressure and/or density. After this recognition, composition programming became the primary control parameter. Lower temperatures, even subcritical temperatures, became common. Pressure (or density) became a secondary control variable.

Modern packed column instruments use multiple, high pressure, reciprocating pumps, operated as flow sources, and independent control of system pressure through the use of electronic back pressure regulators. Such a configuration allows accurate, reproducible composition programming, while retaining flow, pressure and temperature control. Unfortunately, it is still not widely appreciated that a great deal of subtlety is required to pump the fluids used. Many still believe that almost any reciprocating pump can be used with a pump head chiller to make an SFC pump. While most HPLC pumps can be set to compensate for the compressibility of the fluids, the range of compressibility compensation is too small to deal with the fluids most often used in SFC. To attempt to minimize the compressibility range required, the pump is usually chilled to insure the fluid is a liquid, far from its critical temperature. Chilled fluids are dense but are still much more compressible than the normal liquids used in HPLC. To control flow accurately, the pump must have a larger than expected compressibility compensation range. Further, since the compressibility changes with pressure and temperature, the pump must be capable of dynamically changing compressibility compensation. Inadequate compensation results in errors in both the flow-rate and the composition of modified fluids.

From 1979 to 1983, a group at Hewlett-Packard used a modified HPLC with two high-pressure reciprocating pumps operated as flow sources. One pump delivered compressible fluids, while the other was usually used to pump modifiers. A mechanical back pressure regulator controlled downstream pressure. This instrument used a single compressibility compensation, regardless of the fluids used. The compressible fluid and the pump head were cooled to −20°C. The delivery of carbon dioxide, expressed in g/s, varied with pressure and flow-rate. The other pump delivered accurate flows of modifier regardless of pressure and flow. Thus, at different pressures and

flows, the combined pumps delivered different compositions, although the instrument setpoints remained constant. Although not well documented, calibration curves were required to determine the actual, as opposed to set, composition and flow.

Widmer and coworkers at Ciba Geigy, in Basel, Switzerland [74–78] published details of instrument developments through the 1980's. Some of their early work with pressure programming, exhibited the same problems as the earlier Hewlett-Packard approach. The SF pump relied on pump head chillers to minimize differences in compressibility between carbon dioxide and normal liquids used. However, these pumps had inadequate compressibility compensation and produced unrecognized, and undocumented composition and flow programs, which were attributed to pressure programs.

Later this group developed pumps that used expanded compressibility compensation values that used precalculated "look up" tables to optimize pumping based on the actual pressure and temperature. The combination of chilled pump head and compressibility look-up tables largely eliminated variations in flow and composition in the compressible fluid flow as a function of pressure. Another group at Hewlett-Packard, dynamically calculated compressibility factors using equations of state for each fluid used. For accuracy, calculations require precise knowledge of the pressure, temperature and the actual volume being compressed. Leaks, such as through pump check valves, or around pump main seals, effectively increase the volume that needs to be compressed. Pump control algorithms were developed to empirically optimize the calculated nominal compressibility compensation for any fluid, even unknown fluids, plus compensate for leaks. Such compensation minimizes baseline noise, making detector noise levels similar to HPLC.

The compression stroke of the pump also results in adiabatic heating of compressible fluids. If a fluid is heated, it tries to expand, and either its density drops or its pressure rises. If the pump fails to compensate for this heating, the actual flow (and composition delivered) periodically varies during each pump stroke. The heated fluid first delivers excess flow, but as it cools, the flow drops below the set point. Such behaviour causes the double "S" shaped deviations usually observed in the amplified baseline in HPLC. Algorithms producing variable speed control of the pump motor can compensate for such adiabatic heating, and eliminate this periodic variation in both HPLC and SFC.

There have been numerous attempts to generate very low flow-rates of binary fluids for use with either capillary or micropacked columns. For example, switching lines and matched sets of tubing have been used to deliver different concentrations of modifier into a stream of carbon dioxide to make binary mixtures with low flows from a single pressure controlled syringe pump [78]. However, any system using fixed restrictors, and pumps used as pressure sources will produce poor reproducibility.

4.2. Pressure control

For years, it was also universally believed that pressure drop across packed columns produced significant, even "catastrophic", losses in efficiency. It was widely believed [79] that pressure drops could not exceed 20 bar. It was thought that the total efficiency of packed columns could not be >20 000 theoretical plates. This perception arose from limited instrumental capabilities. It is now commonplace to use very long columns (i.e., >2 m long packed with 5 μm particles), with large pressure drops (>150 bar) to generate very high efficiency (i.e., 220 000 plates) [80] compared to HPLC. This reality is in marked contrast to what was almost universally expected, even in 1988.

The use of long columns resulted from a change in control philosophy. Earlier, the pump was used as the pressure controller. The column outlet pressure was not controlled. Long columns produced large pressure drops, and, at modest inlet pressures, the outlet pressure could drop to the point where several (subcritical) phases could exist. The co-existence of several phases destroys chromatographic separations and efficiency. Controlling the column outlet pressure (the pump becomes a flow source, not a pressure source. Consequently, the point in the system with the worst solvent strength becomes the control point. All other positions in the system have greater solvent strength. By controlling this point, problems associated with phase separations or solubility problems at uncontrolled outlet pressures are eliminated.

4.3. Ovens

For many years, it was widely assumed that the fluids used as supercritical mobile phases lost their interesting characteristics when they became subcritical. Subsequently, users strenuously avoided subcritical conditions. There was often a debate about whether reports using binary fluids represented HPLC or SFC. It was implied that subcritical conditions were inherently, dramatically inferior (much lower optimum velocities, much higher pressure drops) to supercritical conditions.

There was a tendency to work at relatively high temperatures to avoid possible phase separations. It is now widely appreciated that the defined state of the fluid is generally irrelevant. Viscosity, diffusion coefficients, density and solvent strength are nearly identical for just supercritical or just subcritical fluids with the same composition. Phase separations almost never occur if the pressure remains high enough.

Ovens used for SFC have traditionally been GC ovens. However, with packed columns, high temperatures tend to be less important, while lower, even sub-ambient, temperatures tend to be more important. This has lead to the use of different kinds of ovens. A maximum temperature of 150°C is not unreasonable. A minimum temperature of less than 0°C is desirable.

5. Separations of acidic and neutral solutes

The real debate about solute polarity in SFC only began in the mid to late 1980's when various workers reported difficulty eluting some modestly polar solutes from capillary columns using pure carbon dioxide as the mobile phase. Curiously, some of the same, or more polar, compounds had been eluted much earlier using packed columns and binary fluids. However, much interest in packed columns has involved attempts to deactivate packing materials, to elute more polar compounds, using pure carbon dioxide.

5.1. Fatty acids and esters

Underivatized fatty acids [64,65,72,81–87] and methyl esters of fatty acids [85] are surprisingly easy to elute using a bonded phase on a silica based packed column and pure carbon dioxide, probably due to the long hydrocarbon tails on the molecules. On the other hand, most aromatic and polysubstituted acids will not elute. Hydroxyl substitution onto a methyl ester of a fatty acid dramatically increased retention [48,88]. The straight chain methyl esters readily eluted from C_{18} columns using pure carbon dioxide but the 2-hydroxyacid methyl esters did not elute.

Lembke and Engelhardt [89] resolved ethyl esters of unusual fatty acids found in plasma that are markers for a genetic disease. They overcame initial selectivity problems by combining three columns in series with three different stationary phases.

5.1.1. Glycerides

The mono-, di- and tri-glycerides are not very polar, possessing no more than a few hydroxyl groups, but have relatively high molecular masses, since they typically contain 30 to 70 carbon atoms. They elute in the approximate order of their molecular mass. The three groups elute separately. They can be eluted with pure carbon dioxide from a packed column but superior selectivity is often obtained using mobile phases consisting of a small amount of hydrogen bonding methanol in acetonitrile, mixed with a majority of carbon dioxide. They are usually eluted from a capillary column using only neat carbon dioxide. The separation of such compounds has been reviewed through 1991 [90]. Five references to packed columns and fifteen references to capillary separations were included. Since then, many more separations have been published. A recent monograph [91] on the use of supercritical fluid technology to characterize and process lipids has appeared.

Most recent interest has revolved around polyunsaturated components in fish oils, which are commercial products. The low temperature of SFC is advantageous in preventing decomposition. Most this work involves the use of relatively high concentrations of polar modifiers and non-polar C_{18} columns.

France et al. [29] compared a Deltabond Octyl column with water saturated carbon dioxide to a polymer based PRN-300 column and a capillary column for the analysis of fatty acids, di- and triglycerides in abused vegetable oils. Mixtures of

acetonitrile and methanol cause significant changes in selectivity. At higher modifier concentrations, elution increases with increasing modifier concentration while selectivity tends to reverse. This may indicate a change to reversed-phase SFC. Lesellier and Tchapla [92] used seven coupled columns and acetonitrile–methanol–carbon dioxide mixtures to resolve a large number of glycerides in rapeseed oil.

5.2. Aromatic carboxylic acids

Smith and Sanagi [66] eluted benzoic acid and benzamide from polymer based polystyrene–divinylbenzene packed column with pure carbon dioxide but peak shapes were poor. This is in accordance with work by Dobbs et al. [6] who measured the solubility of benzoic acid in neat carbon dioxide as 0.25 mol% (200 bar, 35°C), approximately an order of magnitude worse than phenol. They then demonstrated a dramatic improvement in the peak shapes of both when 4.3% methanol was added to the carbon dioxide.

It is often difficult to elute acids from packed columns using pure or even binary fluids. However, there are exceptions than can create confusion in understanding the elution of acids. Gere et al. [93] found that benzoic acid was much less retained than a number of slightly larger peroxides, ethers, esters or alcohols. Benzoic acid was less retained than even benzyl alcohol.

More complex acids are much more difficult to elute (see Section 5.4.1). Since benzoic acid is much less retained than other aromatic acids, it is a poor choice for a probe of acid retention. Clearly, care is required in generalizing based on substitutions near an aromatic ring.

5.3. Phenols

Phenol could be eluted from capillary columns [94] (although with less than ideal peak shapes) using pure carbon dioxide, with pressure programming. In several studies, packed columns [95–97] gave worse peak shapes than capillaries using pure carbon dioxide. None of these studies reported column efficiency, or asymmetry factors, making it difficult to quantify the results.

De Weert et al. [57] systematically used phenol as a probe of silanol activity on numerous silica based packings, including endcapped and polymer deactivated versions, using pure carbon dioxide as the mobile phase. Phenol eluted as a symmetrical peak on all the columns tried. Surprisingly, the addition of methanol to either capillary or packed column mobile phases produced dramatic improvements in the peak shapes of phenols. Mourier et al. [98] extensively studied the effects of a wide range of modifiers. Modifiers included MTBE (a proton acceptor), methylene chloride (strong dipole–dipole), chloroform (proton donor), methanol and acetonitrile. Fifteen different phenols were separated on bare silica, a C_{18} phase and "Pirkle's phase" (for separating enantiomers).

The eleven phenols of US Environmental Protection Agency (EPA) Method 604 were rapidly separated [97] using methanol–carbon dioxide mixtures and various polar stationary phases. The shapes of some of the peaks were poor on some of the columns. The separation was rapid compared to GC, the standard method.

5.4. Stronger acids and tertiary mobile phases

The addition of a second, even weak, functional group to organic acids frequently significantly increases the difficulty in eluting them with binary mixtures. Schwartz [34] separated a group of mycolic acids (C_{48}–C_{60}) using 0.3% formic acid, 0.17% water in carbon dioxide at 100°C, with a micropacked column. The advantage of this mobile phase was its compatibility with FID.

Giorgetti et al. [47] and Datwyler [99] added citric acid to methanol to improve separations of phenols, some weak acids and polymer additives. They [37] systematically studied the effectiveness of a number of organic acids as polar additives. They concluded that, although quite polar, formic acid was ineffective as an additive for these solutes.

In one of the most intriguing SFC papers to date, Anton et al. [100] analyzed complex commercial cremes and suppositories, used as drug delivery vehicles. The products contained preservatives, paraffin's, excipients, perfumes and active ingredients; including parabenes, hydrocortisone, and, in some cases, isomers of crotamiton. After extensive comparisons, the analytical technique of choice was

packed column SFC. Some of the separations required the addition of trifluoroacetic acid, or weaker acids to the mobile phase to achieve decent peak shapes. A significant advantage of SFC involved combining methods. SFC required fewer total analyses per sample for complete characterization.

5.4.1. Poly and hydroxybenzoic acids

Ten hydroxybenzoic acids [50] were nearly baseline separated in less than 2 min using a ternary mobile phase, containing citric acid. In a related paper [48], polycarboxylic acids were separated. The dicarboxylic acids would elute with binary mixtures (methanol in carbon dioxide), but with poor peak shapes. Tricarboxylic or higher substitutions would not elute with such binary fluids. However, the addition of citric or trifluoroacetic acid to the methanol resulted in rapid elution with good peak shapes. Even benzene hexacarboxylic acid will elute with reasonable peak shapes.

The monohydroxyacids have the opposite elution order to the diacids. Some diacids elute before monohydroxybenzoic acids. Salicylic acid, several chlorinated derivatives, plus 3- and 4-hydroxybenzoic acid were eluted from a capillary column [78] using pure carbon dioxide, ethanol modified carbon dioxide and ethanol containing citric acid in carbon dioxide. With pure carbon dioxide, all but one peak tailed severely. The addition of ethanol sharpened the peaks, but some appeared to front. The addition of citric acid changed selectivity. The peaks tended to be rather broad.

5.5. Summary of acidic elution

Many monofunctional acids, in particular fatty acids, will elute from either capillary or packed columns using pure carbon dioxide as the mobile phase. However, the addition of a second polar functionality to the molecule generally causes a lack of elution. Additional acids can be eluted with binary fluids, especially with hydrogen bonding modifiers like methanol. However, a second or third polar functionality on a molecule generally results in poor peak shapes or even no elution. With acidic additives, virtually all acidic solutes, even with multiple acidic substitutions, will elute. In fact, surfactants containing an unhindered sulfonic acid in the repeating unit can be eluted. Dyes with five and six sulfonic acid moieties (also containing multiple hindered amine functionalities) can be eluted with good peak shapes using trifluorosulfonic acid as a additive in methanol–carbon dioxide mixtures.

Basic additives tend to make the peak shapes of acidic solutes worse. Weaker acids than the solutes tend to be ineffective in improving peak shapes. Stronger acids suppress the tailing of acidic solutes best. Steuer et al. [46], and Anton et al. [100] have both used ammonium acetate as an additive to try to suppress the tailing in samples containing both acidic and basic solutes.

5.6. Amides and sulfonamides

Several reports have been published showing that benzamides, and sulfonamides are not eluted with pure carbon dioxide but can be eluted using binary mixtures of methanol in carbon dioxide. Benzamides [59] were shown to be more retained than anilines, and hindered benzylamines, but less retained than primary aliphatic amines. 4-Aminobenzamide was much more retained than primary aliphatic amines, even though the additional amino group is a very weak base.

Combs et al. [101] separated eight regulated sulfonamides. Ramsey et al. [102], Berry et al. [19] and Perkins et al. [103], had all published partial separations of many of these same sulfonamides. Berger [39] had also briefly described peak reversals of several sulfonamides accompanying temperature changes.

In normal-phase HPLC aliphatic amides are much more strongly retained than aromatic amides. The opposite appears to be true in packed column SFC.

5.7. Saccharides

Chester and Innis [104] studied oligo- and polysaccharides, as well as polyglycerol esters [105] using capillary columns and pure carbon dioxide. The polyglycerols, with one to four free hydroxyl groups could be eluted but peaks were broad and tailing, especially with the four free hydroxyl groups. On derivatization, retention decreased and the peaks became much taller and narrower. The degree of improvement followed the number of free hydroxyl

groups covered. These compounds have at least one long hydrocarbon tail.

The polysaccharides would not elute with pure carbon dioxide. The solubility is simply too low [9]. However, on silylation, polysaccharides, with up to 20 repeating units, could be eluted with excellent peak shapes. For polyglucose, with 18 repeating units, 56 hydroxyl groups were covered and the molecular mass of the derivative approached 7000.

Carraud et al. [106] demonstrated a simple separation of two saccharides with light scattering detection in 1987. Herbreteau [107] used carbon dioxide with 8% water in methanol to separate eight sugars in 10 min. Carbohydrate separations by SFC have recently been reviewed [108].

5.8. Steroids

Cholesterol and most ketosteroids can be readily eluted from either packed or capillary columns using pure carbon dioxide. However, if the number of polar substitutions is increased, the ease of elution decreases. Multiple hydroxyl substitutions make it difficult or impossible to elute the four ring steroid backbone. Most capillary separations of steroids have used pure carbon dioxide. Most, but not all (i.e., [109]), packed column separations have used modifiers. The compounds eluted from packed columns with pure carbon dioxide have been mostly the less polar ketosteroids. A review lists publications involving the separation of steroids up to 1991 [90].

Bored et al. [36] also demonstrated the separation of progesterone and estrone using 1.5% methanol in carbon dioxide. Each is a ketosteroid with a single hydroxyl group. These compounds can be eluted in GC. Several rapid separations of polyfunctional corticosteroids have been published. Berry et al. [19] separated eight steroids in less than 6 min using 20% methoxyethanol in carbon dioxide with a 100×4.6 mm I.D. column packed with 5 μm silica. Eleven steroids, including testosterone, estrone, estradiol, cortisone, hydrocortisone and estriol were separated [110] in less than 2 min using 6.1% methanol in carbon dioxide and a 75×4.6 mm I.D. column packed with 3 μm cyanopropyl coated particles. Four hydroxysteroids have been separated [111] in less than 10 s using a 1.5 μm pellicular diol packing in a 3 cm column. Polyhydroxyl steroids [67] have also been used as probes to explore mobile phase-stationary phase interactions.

Henion and coworkers [112,113] identified steroids abused in horse racing in urine and tissue. Both UV and MS–MS detectors were used. Hanson [114] recently studied the retention characteristics of steroids and demonstrated semi-preparative separations using an analytical instrument.

Ecdysteroids [115–117] contain up to seven hydroxyl groups on the four ring steroid backbone. They do not elute with pure carbon dioxide but are easily separated using low concentrations of methanol in carbon dioxide.

Bile acids have the same fused 4 ring structure common in steroids but with an added carboxylic acid functionality. These compounds were rapidly separated [118] using binary methanol–carbon dioxide mixtures. The method was found acceptable for routine quality control of pharmaceutical capsules and tablets.

5.9. Drug compounds and metabolites

Phenylbutazone and major metabolites were separated [119] both from a commercial tablet and from human serum. The benzodiazepams eluted with poor peak shapes using a Deltabond cyano column and pure carbon dioxide [120]. However, they, and their more polar metabolites readily elute with good peak shapes using methanol or ethanol modified carbon dioxide [121–123].

Evans and Smith [124] studied the hydroxylated metabolites of dialkyldithio-carbamates as models for drug metabolites. They concluded that packed column SFC was suitable for the analysis of such compounds with up to three hydroxyl groups, using methanol modified carbon dioxide.

5.9.1. Veterinary drugs

Ramsey et al. [102] used SFE–SFC–MS–MS to detect and quantitate five different veterinary drugs in pigs kidney. These drugs included three di-alcohols: diethylstibestrol, dienestrol and hexestrol; trimethoprim, containing two nitrogens in an aromatic ring, two amino groups bonded directly to the ring and three methoxy groups bonded to another ring; and sulfamethazine. The mobile phase was initially pure carbon dioxide with a near step change

to 20% modifier after 8 min. The peaks eluted starting at 10 min. All the peaks tailed slightly.

Carboxylic polyether antibiotics such as monensin, salinomycin and narasin are added to beef and poultry feeds. These compounds are difficult to detect because they contain only weak chromophores. Berry et al. [17] separated them with an amino bonded phase and 15% methanol in carbon dioxide; and detected them with a light scattering detector.

5.10. Antibiotics

Games [125] presented EI and ammonia CI mass spectra from the SFC–MS elution of the monosaccharide antibiotic lincomycin, using methanol modified carbon dioxide. This compound contains a ternary amino nitrogen in a ring, an amide functionality, plus three exposed hydroxyl groups. More recently [126], the same group used SFC–MS–MS to study the sodium salts of antibiotics in animal feed. Berry et al. [17] eluted penicillin G eluted from an amino stationary phase, using 15% methanol in carbon dioxide.

Lane [127] used packed columns and binary fluids to separate trace contaminants from several antibiotics produced in a fermentation broth. Monosaccharides and disaccharides of Avermectin were separated from contaminants in an extract, and detected by both UV and MS. Similarly, cephalosporin diastereoisomers and another structural isomer were resolved and detected. The identity of a number of minor impurities was established. Finally the antibiotic erythromycin, a disaccharide, was resolved from minor impurities which were identified by MS.

Niessen et al. [128] reported the separation of the antibiotic Erythromycin A, plus several derivatives, using a binary mobile phase of methanol in carbon dioxide. In another reference Niessen et al. [129] detected trace levels of Mitomycin C in plasma samples. Pyo et al. [130] used water to modify carbon dioxide to elute polar antibiotics.

5.11. Natural products

A review article in French [90] summarizes the separation of relatively low polarity, mostly acidic solutes in natural products up to 1991. More than 200 references are given. The solutes covered include triglycerides, fatty acids, steroids, carotenoids and terpenes. Thus, the solutes are mostly low to moderately polar acids in matrices such as vegetable oils, essential oils, plus extracts from urine, plasma and tissue extracts. The separation conditions on both capillary and packed columns are given. In many cases, the capillary separation is accomplished using pure carbon dioxide, while the packed column separation requires the addition of a small concentration of modifier. A few of the articles reviewed used ternary mobile phases containing such additives as water or formic acid.

5.11.1. Vitamins

The first separation of α- and β-carotene by SFC was reported by Giddings et al. [131] in 1968 using pure carbon dioxide. Gere [132] also separated α- and β-carotene, plus lycopene from paprika oleoresin. Later, Skelton et al. [133] performed on line-extraction, followed by packed column SFC on a commercial paprika sample. A review [90] up to 1991 lists five packed column carotene references but not Giddings, Gere, Randall or Skelton. It also lists five capillary separations. Another review covering both HPLC and SFC appeared in 1993 [110]. Most later work has involved the use of modified fluids apparently due to enhanced selectivity. Lesellier has published extensively (i.e., [134]) on carotene. Several other more recent studies have also appeared.

Bored et al. [36] separated ubiquinones and fat soluble vitamins. They stated that neither of the mixtures would elute from C_8 silica based columns using pure carbon dioxide as the mobile phase. Randall [18,42] also reported on the retention characteristics of similar compounds. Subsequently, there have been many reports of both fat soluble vitamins and carotene separations.

5.11.2. Mycotoxins

A family of mycotoxins was separated [135]. These mycotoxins contain ether, ester and keto functionalities, plus one to three hydroxyl substitutions. One mycotoxin contained a secondary amine adjacent to a carbonyl. All were eluted from capillaries using pure carbon dioxide. Peak shapes improved as the stationary phase polarity increased

from methyl, to bi-phenyl, to Carbowax. However, the peaks of solutes containing multiple hydroxyl groups tailed severely. On packed columns, more polar phases gave better separations when methanol modified carbon dioxide was used as the mobile phase. Relatively sharp, symmetrical peaks were obtained. The authors noted that the results on the packed columns were approximately 8-times faster than on the capillary columns.

5.11.3. Glycosides, glycolipids, saccharides

Carbohydrates, glycosides and glycolipids have been separated after silyl derivatization [104,136] on capillary columns using pure carbon dioxide. The separation of saccharides and sugar conjugates in a number of natural products have been demonstrated. The first reports of such separations with mass spectrometric and light scattering detection appeared more than 10 years ago. Games baseline separated eight sugars in 12 min using a methanol program from 8 to 18% in carbon dioxide on a cyanopropyl column and a light scattering detector.

5.11.4. Ginkgolides

Strode et al. [137] reported on the separation of five closely related compounds found in extracts from *Ginkgo biloba* leaves. These compounds have very poor chromophores and were detected using light scattering detection.

5.11.5. Taxol

Taxol is an important new anticancer drug, originally extracted from the bark of an endangered tree. The active ingredients in the extracts are moderately complex, with numerous functional groups. Taxol itself contains three hydroxyl groups, two acetate groups, a benzoate, several keto groups and a secondary ketoamine.

Heaton et al. [138] used SFE to extract the active ingredients and compared capillary and packed column separations. Apolar columns gave poor results. The best separations in the shortest time were obtained using a packed column with both pressure and methanol modifier gradients. Klee and Wang [139] separated taxol from a myriad of other components in both bark extracts and in cell culture extracts using binary mixtures of methanol in carbon dioxide. An example is shown in Fig. 2.

6. Separation of basic solutes by SFC

6.1. Separation using carbon dioxide alone

Capillary SFC was introduced in 1981, and, over the next several years, most of the subsequent separations of bases used capillary columns and pure carbon dioxide. However, primary and secondary aliphatic amines generally did not elute with pure carbon dioxide, precipitating a rather extensive debate about the potential reaction of bases with carbon dioxide and the effect such reactions might have on elution. Since ammonia reacts with carbon dioxide to form ammonium carbamate, it was speculated that the amines might be reacting with the carbon dioxide to form similar insoluble reaction products. In 1954, Francis [4] had reported that several amines, including di-(β-cyanoethyl)-amine and 1-amino-2-methyl-1-propanol formed salts when mixed with liquid carbon dioxide at room temperature and tank pressure.

David and Sandra [140] were unable to elute long chain fatty amines using pure carbon dioxide and a capillary column. Since it was thought that the column activity and retentivity were not a problem (the compounds could be eluted at higher temperature by GC), interaction with the mobile phase was considered more likely. They advocated derivatization with trifluoroacetate, to produce less polar, easier to separate species. Shomburg et al. [141] also speculated on the problems eluting primary and secondary amines using pure carbon dioxide, from both capillary and micropacked columns. They tended to rule out mobile phase problems.

In a paper on capillary separations of aliphatic amines, Gyllenhaal and Vessman [142] stated that the solutes were separated as amines (not as carbamates or some other reaction product) in carbon dioxide. They also substituted nitrous oxide for carbon dioxide and obtained the same partition ratios for a least one amine. The test solutes were primary, secondary, and tertiary octylamine, tributylamine and dihexylamine, plus various substituted analogs of the amino alcohol Metoprolol. Ashraf-Khorassani et al. [143] also replaced carbon dioxide with nitrous oxide in both SFE and SFC, to avoid formation of "insoluble carbamates". The extraction efficiency improved for the bases studied. Both capillary and mi-

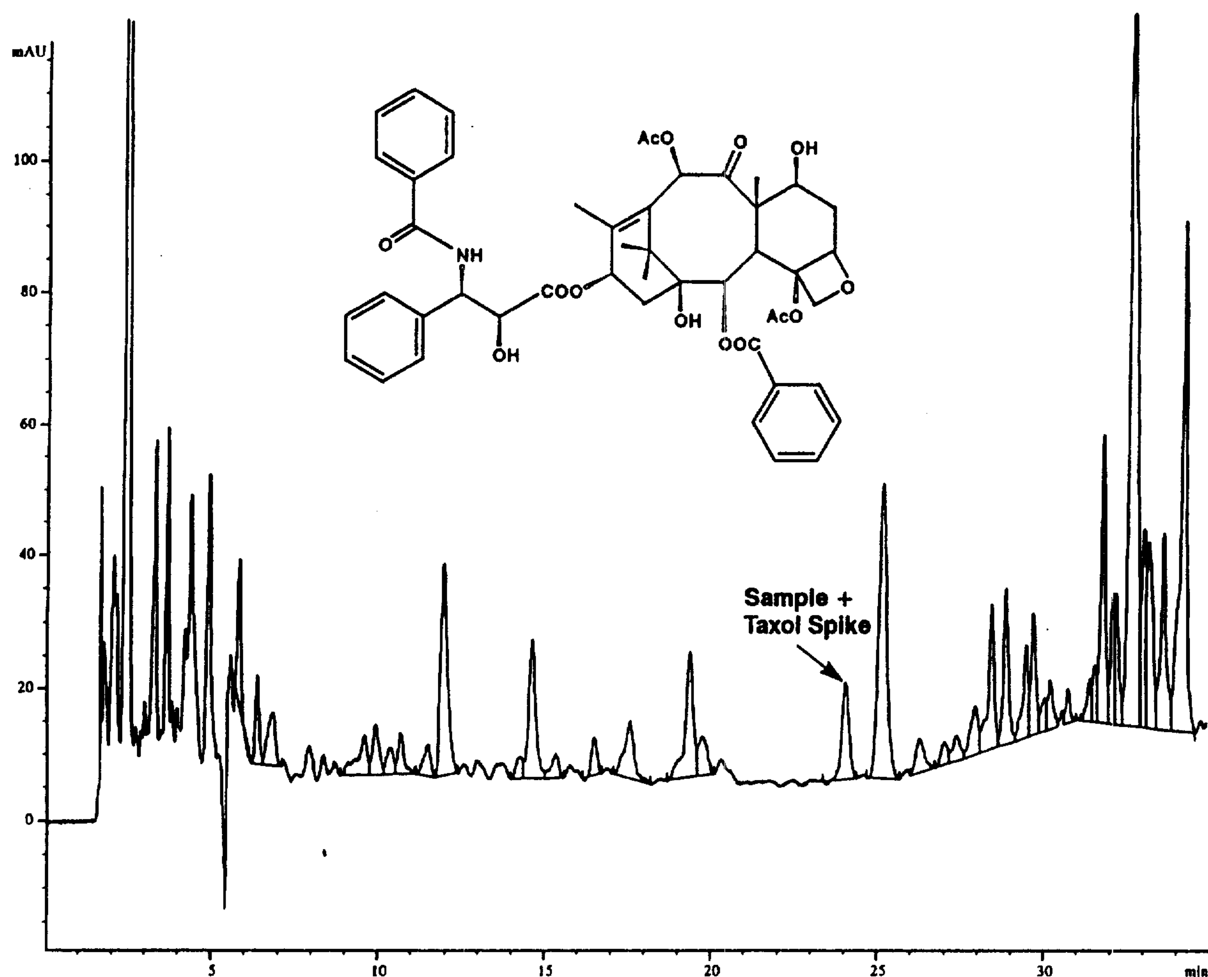

Fig. 2. Separation of taxol from a cell culture extract. 250×4.6 mm, 5 μm Lichrosphere Diol, gradient from 8 to 35% methanol in carbon dioxide, 2 ml/min, 30°C, 150 bar.

cropacked column chromatograms of diphenylamine, naphthol and caffeine were shown using pure nitrous oxide and FID. The caffeine tailed on the micropacked column.

Fields and coworkers [144–146] separated caffeine and theophylline on a capillary column using pure carbon dioxide, but the peak shapes were relatively poor. They suggested two alternate approaches: binary or tertiary mobile phases, or more polar, pure fluids. In addition, they mentioned the possible use of reversed micelles. Although the xanthines differ by only minor methyl substitutions, their solubility in carbon dioxide varies sharply [147]. Caffeine is relatively soluble in pure carbon dioxide, but theophylline is 1/10th, and theobromine 1/100th as soluble. At 199 bar and 40°C, caffeine was soluble to 1.3 g/kg [1300 parts per million (ppm)], or $3\cdot10^{-4}$ mol. Theophylline, under the same conditions, is soluble to 44 ppm ($1.1\cdot10^{-5}$ mol), while theobromine is soluble to only 3.6 ppm (8.8·

10^{-7} mol). Solubility approximately doubles at 350 bar. Solubility approximately doubles again when the temperature is raised to 80°C at 350 bar. At a linear velocity of 1 c/s, well above optimum, the flow through a 50 μm capillary column is 20 nl/s. Since the density in capillary SFC varies from 0.1 to approaching 1 g/cm^3, the mass flow through the column is 2 to 20 μg/s of carbon dioxide. A saturated solution of theobromine would produce a flux in the effluent of a capillary column of 7 to 70 pg/s.

Fields et al. [144] demonstrated the separation of tertiary aliphatic amines on a capillary column with very good peak shapes. Retention followed molecular mass and triethylamine eluted before trioctylamine. Fields and Grolimund [148] also eluted C_{10} and C_{16} straight chain amines with sharp, symmetrical peaks from a methylsilicone capillary column using sulfur hexafluoride as the mobile phase.

In a 1988 report [58] introducing the Deltabond cross-linked cyano columns in SFC, both phenol and 2,6-dimethylaniline showed significant improvement in peak shapes compared to a similar but non-deactivated column, using pure carbon dioxide as the mobile phase. Caffeine gave very sharp peaks using pure carbon dioxide and pressure programming. Several other weak bases were also separated with acceptable peak shapes. Ternary aliphatic amines could not be eluted from standard packings using pure carbon dioxide. However, with the Deltabond column, the authors were able to elute several tertiary aliphatic amines with pure carbon dioxide, but at relatively high temperature and with relatively severe tailing.

Another systematic study in 1990 by De Weerdt et al. [57] found that pyridine, a weak base, failed to elute from silica based ODS packings in micropacked columns, using pure carbon dioxide as the mobile phase. Since pyridine did not elute, the experiment was changed to study the elution of apparently weaker bases such as aniline, N-methyl- and N,N-dimethylaniline, plus several polycyclic aromatic hydrocarbons, all of which would elute. The peaks of all the anilines tailed significantly on all the phases studied. Curiously, the authors compared peak shapes in SFC using pure carbon dioxide to peak shapes in HPLC using a acetonitrile–water (50:50) mixture. Several in-situ deactivation schemes were tried in an effort to improve peak shapes, including injections of trimethylamine (TMA) in water. Although retention times decreased significantly, they drifted with time due to "slow release of TMA".

A toxicological data base has been published [120] listing the relative retention of more than 100 compounds on a Deltabond cyanopropyl micropacked column using pure carbon dioxide. Many of the compounds were bases, such as heroine and barbiturates. However, some of the compounds included were acids, such as members of the "profen" family (i.e., ibuprofen), salicylic acid, etc. A number of solutes did not elute or eluted with poor peak shapes. These included diuretics and benzodiazepines. In later reports by others, these solutes were eluted with binary (or ternary) fluids.

6.2. Separations using binary eluents

Gere et al. [149] published one of the first separations of bases by carbon dioxide based SFC in 1982, when they separated caffeine, theophylline and theobromine using a packed column and methanol modified carbon dioxide as the mobile phase (there had been many patents on decaffeinating coffee using either pure carbon dioxide or water–carbon dioxide mixtures, issued earlier). Aqueous samples of coffee, tea and soft drinks were analyzed, and the authors suggested that as many as 70 analyses per hour could be performed. In 1983, Lauer et al. [10] reported separating the same substances using a PRP-1 column and neat supercritical ammonia. Surprisingly, significantly more polar bases were not separated for a number of years.

In 1988, Ashraf-Khorissani and Taylor [150] had some success separating aromatic amines on standard packed columns using pure carbon dioxide, although peak shapes were poor compared to either normal-phase or reversed-phase HPLC separations. A wide ranging study by the same authors [151] summarizes the separation of bases on packed columns using pure carbon dioxide.

Recently, it was reported [152] that primary amines react with carbon dioxide to form unstable carbamoyl compounds. Since such compounds elute with symmetrical peaks, exhibiting modest retention characteristics, and high absorbance, these compounds appear to be relatively soluble in neat carbon dioxide.

Games et al. [19,125] published rapid separations of: (a) caffeine, theophylline and theobromine; (b) sulfonamides, (c) carbamates and (d) numerous other secondary amines, particularly from natural products. Packed columns were used with methanol modified carbon dioxide and mass spectrometric detection. Randall [18,42] reported retention information on a wide range of moderately polar compounds like caffeine, xanthine, adenine, fat soluble vitamins, phthalates, amino and nitro PAHs and steroids. Caffeine and its analogs were recently analyzed [153] in various over the counter drugs, as well as in beverages, similar to Gere et al. [149]. The effects of directly injecting aqueous samples was studied in some detail. In addition, changes in selectivity, including peak reversals, through changes in the stationary phase, were documented.

Games [125] eluted the alkaloid reserpine using methanol modified carbon dioxide, although the peak shape was poor and the peak was broad. This compound contains a secondary and a ternary amine, five methoxy groups and an ester.

Feldopine, a calcium channel blocker, containing a secondary amine in a ring was separated [154] using a binary fluid consisting of methanol in carbon dioxide. Detection was via electron capture detection.

The paper by Upnmoor and Brunner [155] is indicative of the general experience of others with packed columns and binary fluids up to 1989. The authors separated a mixture of caffeine, theophylline and theobromine, similar to Gere et al. [149], plus several weak acids such as salicylic acid, using binary fluids consisting of methanol in carbon dioxide.

Thus, in the 7 years between 1982 and 1989, virtually no improvement in solute polarity had been demonstrated using more deactivated columns, various alternative pure mobile phases, or other binary fluids. In the previous year (1988), the first few papers using very polar additives had appeared, but the advantages of such fluids were not yet widely appreciated.

6.3. Strong bases and ternary fluids

A few examples of the separations of relatively polar bases using binary fluids have appeared. However, such separations tend to be exceptions which confuse the overall picture of what is possible. Generally, the separation of bases that are not sterically hindered, and are stronger bases than aniline, requires the use of ternary mobile phases.

Steuer et al. [45] demonstrated ion pairing in supercritical fluids by separating the enantiomers of propranolol and related beta blockers on an achiral column using a chiral mobile phase additive that formed diasteriomer pairs with the solutes. The additives included both a short peptide (as the chiral mobile phase component) and a short chain amine.

A later publication by Steuer [46] was somewhat less clear cut in proving ion pairing. Both an acidic, and a basic additive were mixed into the mobile phase. The peak shapes of basic solutes improved. These improvements were attributed to the acidic additive. However, similar improvements have been achieved using basic additives without the counter ion being present.

6.3.1. Anilines

In a straightforward study, Berger and Deye [16] showed that anilines and toluidines were easily eluted with excellent peak shapes and near theoretical efficiencies from standard packed columns using low concentrations of methanol modified carbon dioxide. Similar results were obtained when Freon-13, or Freon-23 was substituted for the carbon dioxide. Relative retention followed the polarity of the main fluid. The solutes were most retained using methanol modified Freon-13, and least retained using methanol modified Freon-23. There was no obvious difference in peak shapes or elution order between carbon dioxide and the other fluids.

The solutes did not elute or eluted with poor peak shapes using any of the pure fluids, including Freon-23 (fluoroform) which is presently being touted as a more polar replacement for carbon dioxide. Both standard and polymer deactivated columns were used.

The elution order of the anilines was opposite to the reversed-phase HPLC elution order. In SFC using methanol modified carbon dioxide, N,N-dimethylaniline elutes first and aniline elutes last. Note that the lightest solute, which is also the weakest base, eluted last, while the heaviest, strongest base eluted first. This suggests that the nitrogen, not the ring, was interacting with the stationary phase. The

elution order appears to be inversely related to the degree of steric hindrance of the nitrogen atom.

6.3.2. Carbamates

The separation of the carbamates [19,125] was somewhat ironic since there was a contemporary controversy about whether primary amines reacted with carbon dioxide to form "insoluble carbamates" using pure carbon dioxide as the mobile phase. If it was obvious that the "reaction products" were soluble in methanol modified fluids, then it is odd that methanol modified fluids were largely ignored, if not actively avoided during this time period. Instead, the desire to retain pure fluids dominated over practical results. The carbamates of EPA Method 531.1 were efficiently separated in less than 10 min [156] on diol columns using methanol modified carbon dioxide. UV and nitrogen–phosphorus (NPD) detection were used simultaneously. Aqueous samples were pseudo on-line extracted on a pre-column which was dried before injection.

6.4. Separation of strong bases and the use of ternary eluents

6.4.1. Benzylamines

Several papers [15,59] used benzylamines as probes for studying the effects of additives on the separation of strong bases. Benzylamines are approximately four orders of magnitude more basic than anilines. Benzylamines will generally not elute from packed columns, or will elute with extremely poor peak shapes, using binary fluids. The addition of some additives, dramatically improves peak shapes. Tetrabutylammonium hydroxide added to the methanol significantly improved peak shapes. Isopropylamine, a weaker but less sterically hindered base, was even more effective in improving peak shapes. It was concluded that primary aliphatic amines were the most effective additives. For virtually all organic bases, including the benzylamines, the addition of 0.5% of a short chain aliphatic amine to methanol produced excellent peak shapes and high efficiency.

Common additives used in HPLC, such as trimethylamine and triethylamine, were ineffective additives. Polyfunctional amines such as ethylenediamine and diethylenetriamine caused peak shapes to degrade. Trifluoroacetic acid, hexanoic acid and the sodium salt of octadecylsulfonate, all widely used ion pairing agents, were completely ineffective in improving peak shapes. In a few cases, the acid appeared to degrade peak shapes further.

In the elution of the benzylamines, steric hindrance was found to produce similar results to those reported for anilines. A weaker, primary benzylamine will elute much later than a stronger secondary or ternary amine.

Phenylethyl amines [59] tend to be more basic than benzylamines. Since the amine functionality tends to be less shielded by the ring than the benzylamines, the phenylethylamines tends to be more retained.

6.4.2. Amino acids and derivatives

A few PTH amino acids were separated [125] using modified carbon dioxide. A much more extensive group of PTH amino acids were the subject of several later reports [44,157], using quaternary ammonium salts added to methanol. This addition expanded the range of PTH acids that would elute. One of these papers represents the first published use of tertiary fluids in SFC.

FMOC derivatives of amino acids have also been separated [158].

6.4.3. Analysis of basic drugs

6.4.3.1. Imidazoles

A wide range of imidazole derivatives have been separated [159] using a complex mobile phase containing small amounts of methylamine and water in carbon dioxide.

Multifunctional bases were separated [160] using low concentrations of tetrabutylammonium hydroxide in methanol as modifier. The compounds included: pseudoephedrine, an amino alcohol; mercaptopurine and trimethoprim, both containing four nitrogens; and zidovudine (AZT), containing five nitrogens. Hedrick and Taylor [161] demonstrated on-line extraction, followed by SFC elution of pseudoephedrine and triprolidine, from aqueous samples. The mobile phase in both cases included tetrabutylammonium hydroxide, since both solutes are bases.

The relative retention of omeprazole and four

analogs, including a sulfoxide and a sulfone was studied on six different packed column stationary phases, using a tertiary mobile phase of carbon dioxide, methanol, and triethylamine [162]. In related work, Gyllenhaal and Vessman [163] also reported on the effects of modifier identity, amine additive identity, and concentration on the retention and selectivity of alkylated omeprazoles.

Smith et al. [164] eluted ranitidine (Zantac) and its polar metabolites by adding methylamine and water to methanol, then mixing with carbon dioxide.

6.4.3.2. Anticonvulsants, antidepressants and stimulants

In 1986, Crowther and Henion [165] eluted caffeine, as well as codeine and cocaine using methanol modified carbon dioxide with a silica based packed column. Numerous basic opium alkaloids were separated [166] from poppy straw extracts using a mobile phase consisting of carbon dioxide, methanol, triethylamine and water.

Three papers in a series [167–169] addressed the separation of phenothiazine anticonvulsants [167], tricyclic antidepressants [168] and stimulants [169], all of which are basic drugs. Most required a basic additive in the mobile phase in order to yield sharp peaks and high efficiency. Reserpine which previously gave a broad tailing peak with methanol modified carbon dioxide [125], produced a narrow, symmetrical peak [167] when the additive was included in the mobile phase. The effect of modifier concentration, temperature and pressure were studied. Changing the modifier concentration was most effective for significantly changing retention. Changing the temperature often (but not always) caused significant changes in selectivity. Pressure was a secondary control variable, only modestly changing either retention or selectivity.

6.4.3.3. Purines and pyrimidines

Purines and pyrimidines were separated [170] on a short diol column using a ternary mobile phase consisting of 10% (methanol containing 0.6% isopropylamine) in carbon dioxide. Some, like guanadine, were only marginally soluble in pure methanol, normally requiring an aqueous or even ionic buffer as solvent. Such compounds define an upper limit to the present state of solute polarity accessible by SFC based on carbon dioxide.

6.4.3.4. Miscellaneous

Salbutamol, a bronchodilator, was separated from six impurities using a Diol packed column and a tertiary mobile phase of carbon dioxide, methanol and *n*-propylamine. The authors report [171] the separation and detection of 1 μg/ml impurities in 1000 μg/ml of the parent in degraded commercial tablets of the drug.

Bailey et al. [172] studied beta blockers on several columns. Ternary mobile phases containing triethylamine gave the best results.

Mono and diamides [173] were eluted using carbon dioxide, methanol and ammonium acetate. A fluorinated amide containing three nitrogens in a ring was separated from mice feed pellets and eluted using packed column SFC with a ternary mixture of carbon dioxide, acetonitrile and methanol.

6.5. Summary of basic separations

Virtually all small molecule (i.e., less than 2000 molecular mass) organic bases can be solubilized and eluted using binary or ternary mobile phases based on carbon dioxide. Anilines are relatively soluble in neat carbon dioxide but can be difficult to elute chromatographically using neat carbon dioxide as the mobile phase. Low concentrations of an organic modifier produce good peak shapes. More polar, aliphatic amines require a basic additive in the mobile phase. The addition of a strong base to the mobile phase often produces dramatic improvements in peak shape. Acidic additives typically make peak shapes worse. There is little evidence that ion pairing works.

To date, the best peak shapes and the shortest analysis times are obtained using conventional silica based columns and ternary mobile phases. Strong bases have not been eluted from capillaries with pure carbon dioxide. Strong monofunctional bases have been eluted from heavily deactivated, polar packings in micropacked columns using pure carbon dioxide, but tail. Strong bases can be eluted from polymer based columns using binary methanol modified carbon dioxide, but tail.

Steric hindrance appears to be extremely important

in the elution of bases. A ternary aliphatic amine will elute earlier than a primary aniline even though the latter may be four orders of magnitude weaker as a base. The addition of a second polar functional group dramatically increases the retention of solutes but polyfunctional amines can be eluted.

7. Chiral separations

The separation of enantiomers is an important new area of separation science. Packed column SFC is finding a significant role in this field and is largely supplanting normal-phase HPLC. The vast majority of chiral separation by SFC have been performed using packed columns and binary or ternary mobile phases. The same columns as used in HPLC are usually used in SFC. The first demonstration of chiral separations was performed in 1986 by Mourier et al. [98].

Bargmann et al. [174] reviewed the application of packed column SFC to the chiral separation of enantiomers through 1992. Macaudiere and co-workers [175,176] reported the enantiomeric separation of a wide range of secondary amides using binary mobile phases. Pirkle recently reviewed chiral separations by SFC [177]. A Cyclobond I column was said [178] to be substantially easier to use in SFC than in HPLC. The authors state that SFC can be used as an alternative to the reversed-phase or polar organic modes used with this column in HPLC. They also stated that SFC is faster.

7.1. Binary fluids

Gasparrini et al. [179,180] reported the resolution of both a secondary aminoalcohol and a primary aminoketone, using a binary mobile phase of methanol–carbon dioxide. They demonstrated extremely rapid (<1 min) separations with relatively high (>baseline) resolution. These authors tend to derivatize highly polar compounds in order to elute less polar entities with binary mobile phases. Stringham et al. [181] reported the resolution of enantiomers of several aminoalcohol intermediates and a final product by both HPLC using ethanol–hexane mixtures and by SFC, using ethanol–carbon dioxide mixtures. The main conclusion was that the Chiracel-OD column gave much higher efficiency in SFC, compared to HPLC, which significantly improved two of the four separations discussed. Enantiomers of amino acid derivatives have been separated [182–184].

Wang et al. [121,122] separated a wide range of metabolites of camazepam, oxyazypam and other benzodiazepines using ethanol–carbon dioxide mixtures. In the chiral separation of camazepam and five metabolites, a cyanopropyl column inserted in front of the Chiracel-ODH column allowed the near baseline resolution of the six pairs of enantiomers in just over 14 min. The separation of a metabolized sample is presented in Fig. 3. Smith and Ma [185] reported the separation of the enantiomers of a number of benzodiazepines; including temazepam, lormetazepam and lorazepam. A number of factors were studied. It was stated that a rigid planar structure resulted in greater enantioselectivity than flexible aliphatic side chains.

Stringham and Blackwell [186] empirically confirmed the concept of isoelution temperatures and peak reversals with temperatures using binary mobile phases on packed columns.

7.2. Ternary mobile phases

A significant fraction of the chiral SFC separations reported in the literature have required tertiary mobile phases. Such findings are not surprising since most chiral separations are performed in the pharmaceutical industry, and a large fraction of drugs, drug intermediates and metabolites are aliphatic and/or polyfunctional amines. The second report published using polar additives in SFC [45] (1988) employed a chiral short peptide and a short chain amine as additives in the mobile phase. This mobile phase allowed the resolution of the enantiomers of the Beta Blocker, propranolol and related compounds on an achiral column.

Bargmann-Leyder et al. [187] developed chiral stationary phases based on tyrosine. They demonstrated separations of hydroxyl and methyl esters of amides. In many of the separations, the addition of low concentrations of acetic acid were required to insure good peak shapes.

Trietz et al. [188] compared SFC and HPLC for the separation of three probe molecules. They extensively studied the effects of chromatographic con-

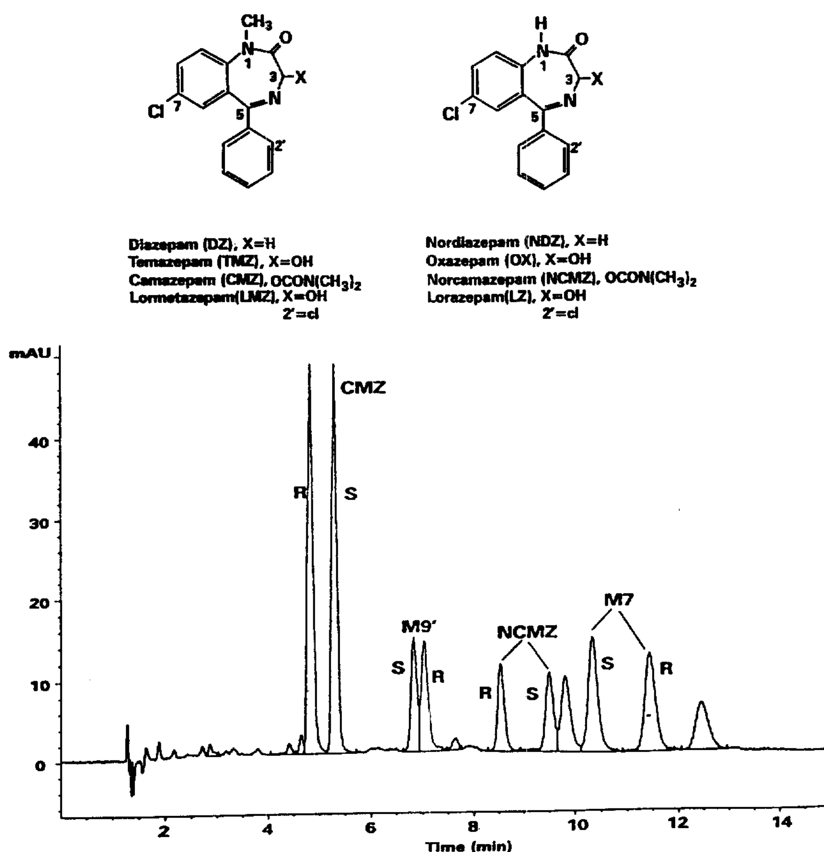

Fig. 3. Chiral separation of camazepam and metabolites. 250×4.6 mm, Chiracel OD-H, 2 ml/min, 10% to 18% ethanol in carbon dioxide, 30°C, 200 bar.

ditions on the separation of the enantiomers of: oxazepam; binaphthol and DHP (pyridine substituted with a carboxylic acid, a nitrophenyl and an ester). All required a binary mobile phase. The DHP was used with ternary mobile phases, but there was little need for the additive. Retention, resolution and selectivity were essentially unchanged when either TFA or acetic acid were added to the mobile phase. Peak shapes were adequate with the binary fluid.

Ohlen et al. [189] separated the enantiomers of a dihydroxypyridine (H324/38), an experimental high blood pressure drug, and its three major metabolites. Some of the metabolites are acids, and required the addition of tartaric acid to the mobile phase. Two different columns were required in series to separate all the enantiomers in a single run.

Gyllenhaal and Vessman [190] quantified the impurities of metoprolol, an aminoalcohol, at the 0.1% level. They normally used a low concentration of triethylamine to improve peak shapes but also report that an excess of acetic acid over the amine improved selectivity.

The enantiomers of chiral antimalarial agents were resolved [191] on an (*S*)-naphthylurea stationary phases using methanol–carbon dioxide mixtures containing a low concentration of triethylamine. These compounds are amino alcohols. Without the amine in the mobile phase, peaks remained significantly tailed. With the additive, peak shape improved. It was noted that modifier concentration had little affect on selectivity. Temperature had a moderately significant effect on selectivity. Other separations of aminoalcohols [45] used a ternary mobile phase.

The effect of six different basic additives on the separation of numerous beta-blockers (aminoalcohols) with a ChyRoSine-A column, was studied in detail [192]. These studies included the effect of the concentration of additive on retention, selectivity and resolution. The most effective additive was *n*-propylamine, a primary aliphatic amine. The authors reported that NMR results indicated a rigid complex was formed between one of the enantiomers and the carbon dioxide, improving chiral recognition. Related results [193] on beta blocker separations, using *n*-propylamine as an additive, were reported in another study. Limits of detection of 2:1000 (with $S/N>5$) were reported.

In another study [194], most beta blockers were separated using binary fluids. Nadolol, with two chiral centers gave only three peaks on a Chiracel OD column in both HPLC and in SFC. Subsequent work in HPLC nearly baseline resolved all four enantiomers, but from an α-AGP column. The enantiomers were only resolved (by HPLC) when the octanoic acid additive was replaced with tetrabutylammonium bromide. There was no attempt to use either acidic or basic additives in the SFC mobile phase. In unrelated work, Wilson [195] was able to nearly baseline resolve all four Nadolol enantiomers on a Chiracel OD column by including 0.5% isopropylamine in the methanol modifier and programming composition to allow separation of the first previously unresolved pair.

In another paper [196], additional NMR evidence was presented which questioned whether there was a reaction between basic solutes and carbon dioxide. It had earlier been suggested [192] that carbon dioxide reacted with one drug to give a more rigid, easier to resolve enantiomer.

The separation of a number of bases was demonstrated [196] along with the effects of mobile phase identity, composition, pressure and temperature. In the course of this study, several compounds were found which did not resolve in SFC but resolved on the same column in HPLC. In SFC, an isopropanol–carbon dioxide mixture failed to resolve phenylalaninol (an aminoalcohol) on a Chiracel OD column. In HPLC, the successful mobile phase consisted of hexane, and isopropanol, and either 0.1% trifluoroacetic acid (TFA), or 0.1% diethylamine.

In retrospect, the phenylalaninol peak was rather broad. As in the Nadolol example [194,195], another aliphatic amine, as a mobile phase additive, should dramatically narrow such broad peaks and might even have changed the SFC result [15,59].

Biermanns et al. [197,198] separated beta blockers on a Chiracel OD column and demonstrated dramatic peak shape, and subsequently, resolution improvements through the addition of a polar additive (isopropylamine) to the mobile phase. They demonstrated four orders of linear response between 0.25 and 2500 ppm propranolol, with quantitative R.S.D.s for high concentrations as low as ±0.02%.

Another example [196] contained three nitrogens, the most active of which was a slightly hindered aliphatic, secondary amine. In SFC, a single broad peak was observed at 10 min, but no additive was included in the mobile phase. However, an amine additive was included in the HPLC mobile phase and the enantiomers were resolved even though the peaks were very broad. The results suggest some caution should be applied to insure an "apples to apples" comparison between the two techniques. In light of the previous reports [194,195], the absence of an additive in the SFC mobile phases but their presence in the HPLC example suggest an "apples to oranges" comparison may have been made.

Wilson [199], separated the enantiomers of acidic flurbiprofen, and ibuprofen, using binary fluids. In a second paper, he also separated the alcohol Warfarin [200] and other compounds using binary mixtures of methanol in carbon dioxide. No additive was needed. Some weakly basic solutes, such as sulconazole, metobarbital, ethosuximide and the sulfonamide; bendroflumethiazide, could also be separated using the binary mobile phase of methanol in carbon dioxide. However, more basic solutes such as buclizine, and indapamide; and the aminoalcohols: propranolol and hydroxyzine, required a tertiary mobile phase of isopropylamine, methanol and carbon dioxide.

Terfloth et al. [201] reported the separation of 8 members of the "profen" family, as well as other compounds like warafrin, benzoin, and others using both acidic and basic additives on a new brush type CPS incorporated into a polysiloxane immobilized on silica gel (poly Welch-O). They report improved column efficiency and reduced analysis time compared to HPLC.

Subsequently [202], this new phase was applied to a wide range of separations by both HPLC and SFC. In this paper, some 120 different separations were reported but it appears that more than 1200 separations have been successfully performed [203].

A thorough comparison [204] of chiral separations by HPLC and SFC was made using Chiracel AD and OD and several mobile phases. The authors concluded that there can be marked discrepancies in selectivity between HPLC and SFC using these cellulose and amylose derived stationary phases. They recommend trying both HPLC and SFC to find the best selectivity for each new application.

Lynam and Nicolas [205] compared SFC and HPLC using a Chiracel OD column. They obtained remarkably similar separations, except the efficiency was 3- to 5-times higher in SFC compared to HPLC. CBZ-Phenylalaninol was eluted with binary mixtures but CBZ-Alanine required the addition of TFA to either the HPLC or SFC mobile phase.

Maftouh [206] recently presented a decision tree used by Sanofi in France to develop chiral methods. This is a formal expert system approach to minimize method development time. Candidate compounds are divided into three groups. Polar/ionizable/ionic compounds that are water soluble are processed by reversed-phase HPLC with up to five different carefully chosen stationary phases. All non-polar compounds that dissolve in dichloromethane are processed by SFC, with a different set of five columns. Medium polarity molecules, soluble in methanol, isopropanol or acetonitrile, may be processed by both SFC and HPLC to determine the best approach. Further decision trees were presented to differentiate which columns were most likely to separate the enantiomers of interest. Several SFC chromatograms were presented, including at least one using additives. One drug contained two chiral centers, but no single column separated both enantiomeric pairs. A combination of two different columns in series provided baseline resolution of all four isomers.

Mathre [207,208] of Merck (Rahway, NJ, USA) presented several posters about the SFC separation of enantiomers in process research into the synthesis of new drugs. In one synthetic path [207] a methyl ester, a ketosulfone, a hydroxysulfone and a the final product containing a sulfone, a sulfonamide and a secondary amine were each separated into their corresponding enantiomers on different columns.

The hydroxysulfone had two chiral centers. The four resulting enantiomers were all baseline resolved in less than 15 min, with the Chiracel AD column exhibiting up to 12 500 plates. Since the particle size is 10 μm, this represents the maximum theoretical efficiency expected from an achiral column. Others have reported similar dramatic improvements in efficiency compared to HPLC using the same columns.

The final product (MK-0507) required the addition of isopropylamine to the mobile phase to ensure narrow symmetrical peaks. The solute can exhibit *cis-* and *trans-*isomers, each one of which has a chiral center. None of the chiral columns tested could resolve all four isomers. However, an achiral Inert Si column in front of either a Chirapac AD or AS column separated all the isomers.

In another paper [208] presented at the same meeting, Mathre et al. demonstrated a validated method for the determination of the steriochemical purity of another drug with two chiral centers. This drug (MK-0499) contained a cyano, a hydroxyl, a nitrogen in an aliphatic ring and a methyl sulfonamide functionality. Baseline separation was achieved by (in the authors words) "a combination of brute force" (three Chiracel OJ columns in series) "and finesse" (a slow methanol–isopropylamine modifier gradient). Minor components were quantified at 0.5% with an R.S.D. of 4.88%. The major component was quantified at 99.5% with an R.S.D. of 0.03%. The limit of detection of the minor component was ca. 0.1%. In another case, a silica column in series with a Chiracel OD-H column resolved the four stereoisomers present.

Blum et al. [209] resolved a number of acids and poly alcohols by both HPLC and SFC using a new Pirkle Type stationary phase. In many cases, trifluoroacetic acid was added to the mobile phases used in both HPLC and SFC. This was particularly true for the separation of CBZ-analine and TN-mesitylsulfonyl-alanine.

Sandra and coworkers [210–212] and Kot [213] have used as many as five different chiral stationary phases in series, to attempt to produce a pseudo "universal" phase. The enantiomers of totally unrelated solutes have been successfully eluted in a

single run using this approach. However, Pirkle has pointed out that most of the analysis time is wasted in such an approach, since most of the columns add nothing, and can even be detrimental, to the separation. Further, the optimum conditions for one pair of enantiomers on one of the columns is unlikely to produce optimum conditions for a different pair on one of the other columns. It remains to be seen whether such an approach will persist.

8. Present limits, future directions

The next horizon for SFC involves the elution and separation of molecules containing both strong primary aliphatic amines and an unhindered carboxylic acids (zwitterions). In earlier sections of this review, it was repeatedly pointed out that acidic solutes generally require acidic additives and basic solutes require basic additives. Clearly, it should be difficult to suppress both strongly acidic and strongly basic functionalities at the same time. Molecules containing such combinations constitute the current cutting edge of SFC separations using carbon dioxide based fluids. However, this region is almost unexplored.

Amino alcohols, and amino acids are relatively easily eluted and separated. Dyes with multiple sulfonic acid groups, which are also hindered amines can be readily eluted using a mobile phase consisting of a low concentration (<1%) of TFA or trifluorosulfonic acid in methanol added to carbon dioxide. A chromatogram, showing the structures of several blue dyes is presented in Fig. 4.

Underivatized amino acids have been separated [214,215]. In one report [214], the mobile phase consisted of fluids modified with pyridine–methanol–water and triethylamine or, alternately substitut-

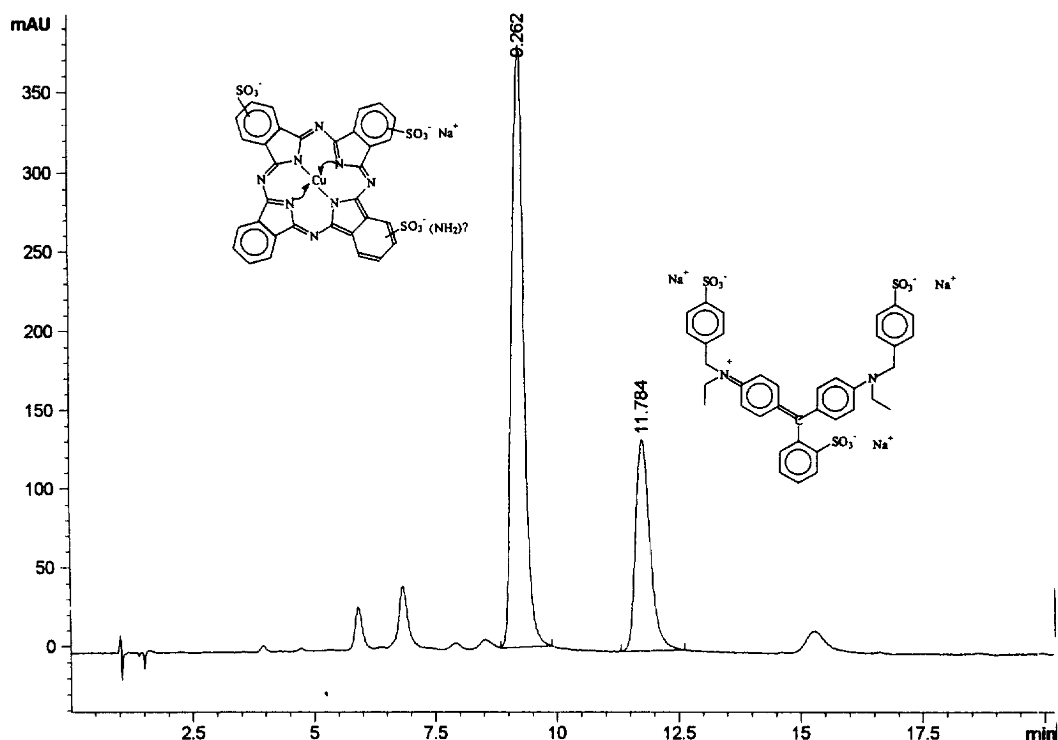

Fig. 4. Separation of polar dyes containing sulfonic acid and amine functionalities; 250×4.6 mm, 5 μm Lichrospher Cyano, 2 ml/min, 30% methanol (containing 0.5% trifluorosulfonic acid) in carbon dioxide, 40°C, 150 bar.

ing ethylene glycol for the pyridine. Detection was via a light scattering detector.

Lane [127] resolved and detected diastereoisomers and another structural isomer of the zwitterion cephalosporin.

There has been some successful work with short peptides but it remains unclear to what extent SFC can be enhanced for separating such compounds. Berry et al. [17] eluted a short peptide containing three amino acids. The peptide was eluted using chlorodifluoromethane ($CHClF_2$) with 10% methanol (containing 0.5% TFA). Steuer et al. [45] used a short peptide (as the chiral mobile phase component) and a short chain amine as additives to separate the enantiomers of the aminoalcohol propranolol and related beta blockers on an achiral column.

One limitation in solute polarity appears to relate to solubility of very polar compounds in the mobile phases used. A good rule of thumb [216] holds that substances with some solubility in methanol or a less polar organic solvent can likely be separated by carbon dioxide based mobile phases. As a corollary, substances requiring aqueous conditions or aqueous, ionic buffers to dissolve are, at present, poor candidates for separation by carbon dioxide based SFC.

SFC is a poor choice for most biopolymers such as larger peptides, proteins, DNA, RNA, etc. On the other hand, Weder [217,218] showed that the activity of enzymes like lysozyme and ribonuclease was unaffected by exposure to carbon dioxide at 300 bar and room temperature. Randolph et al. [219] showed that alkaline phosphatase was active in carbon dioxide modified with 0.1% water. Cholesterol oxidase was also active [219] in supercritical carbon dioxide. There are lipophylic proteins that have not been evaluated as solutes.

Lesillier's apparent recent demonstration of reversed-phase SFC with modest polarity solutes also raises intriguing possibilities. Enhanced fluidity HPLC is instrumentally identical to packed column SFC. However, the instrumental transition region between enhanced fluidity HPLC and SFC is almost unexplored. Is there a more polar operating region of reversed-phase SFC?

Acetic and formic acid are completely miscible with carbon dioxide [3,4] but have never been used as the primary modifier at high concentrations Typically acids are added to no more than 1% in methanol which is then used as the modifier. Since carbon dioxide based fluids are nominally non-aqueous, such fluids should not be excessively corrosive. The addition of water to binary fluids has been only marginally evaluated. Some fluorocarbons have dramatically higher dipole moments than carbon dioxide. Used neat or modified, they may extend the range of solutes that can be solubilized and eluted.

Another possible way to overcome the low polarity of carbon dioxide, or even binary or ternary mixtures of carbon dioxide, is the creation of reversed micelles. In a reversed micelle, the apolar hydrocarbon tails of a surfactant are arranged on the outside of a sphere, while the polar heads are pointed toward the interior. Polar fluids and solutes can be contained inside a small region of high polarity. Most of the work on reversed micelles has involved extractions of very polar solutes, including biopolymers, from polar matrices. Amino acids have been transported in reversed micelles formed in ethane and propane [220]. More recently [221], micelles were formed in carbon dioxide through use of a fluoroether surfactant that supported the transport of proteins in an internal aqueous environment.

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