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# Chiral separations by packed-column super- and subcritical fluid chromatography

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ABSTRACT

The higher diffusivity and lower viscosity of supercritical and near-critical fluids can make packed-column supercritical fluid chromatography or subcritical fluid chromatography (SubFC) faster with improved resolution over normal-phase HPLC for chiral separations. However, superior fluid characteristics do not guarantee enhanced resolution or shorter analysis time. In the case of phenylalaninol, a $^1$H NMR spectrum indicates interaction of the solute with carbon dioxide. Such interaction may explain the poorer resolution achieved by SubFC than by HPLC. For a secondary amine, its $^1$H NMR spectra showed no shift while poorer resolution was again observed for SubFC when compared to HPLC. Thus, the NMR data by itself do not conclusively indicate either reaction between basic solutes and carbon dioxide or the likelihood of chiral resolution.

The effects of column outlet pressure, organic modifier composition, pump flow-rate and column temperature for hydroxyzine were studied. Of the physical parameters studied, modifier composition has the greatest impact on retention. Increasing retention generally increases resolution. Changing temperature generally has less impact on retention but produces the greatest selectivity changes.

## INTRODUCTION

The use of super- and near-critical fluids for chromatography is gaining greater recognition. With recent developments in hardware, the user can now run packed columns with full control over the many chromatographic parameters. In particular, the ability to dynamically mix polar modifiers with highly compressible fluids has dramatically increased the range of solute polarity amenable to separation. In addition, independent control of pressure and flow-rate has made it much easier to interpret results.

Because of the lower viscosity and higher diffusivity of analytes in these mobile phases, packed-column supercritical fluid chromatography (SFC) typically provides a three- to five-fold reduction in analysis time over HPLC [1] and up to a 25-fold decrease in analysis time over capillary SFC [2]. These speed advantages also exist with subcritical fluid chromatography (SubFC) on packed columns.

One of the rapidly growing areas for separation science is chiral resolution. The need to determine enantiomeric purity is of major importance to the pharmaceutical industry. A recent publication [3] indicates that all drugs containing chiral centers must have chiral assays during toxicological and pharmacological testing.

Several groups have previously examined packed-column SFC for chiral analyses [4–8]. Enantiomeric resolution can be attained through solute derivatization, chiral mobile phase additives, or chiral stationary phases. The last mode is the easiest as it allows the user the greatest flexibility with the least amount of sample preparation.

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Chiral columns have been grouped into three categories: functionalized silica gel, polymeric-type and protein-type columns [9]. Among the first category, the Pirkle-type columns are comprised of chiral moieties bound to silica gel that establish individual recognition interactions with the solutes. In contrast, the polymeric columns consist of either an achiral functionalized cellulose, acrylamide or methacrylate, usually coated on silica gel. These types of columns rely on the chirality of the polymer backbone and the polymer geometry to achieve chiral discrimination. Columns that fall in between polymeric and protein phases include the crown ethers and the cyclodextrin phases. The commercially available protein columns typically employ bovine serum albumin, $\alpha$ acid glycoprotein or ovomucoid phases to recognize and separate chiral compounds.

This work focuses on the investigation of the cellulose-coated polymer-type columns, especially Chiralcel OD. Packed-column SFC typically provides faster resolution than normal-phase HPLC. In addition, packed-column SFC allows the analyst to alter the pressure, composition, flow and temperature to achieve separation. The effects of these control variables on chiral resolution are studied. The speed of analysis and re-equilibration of the chromatographic system allow the user to evaluate several columns for chiral resolution in a very short time. Examples of this speed are demonstrated.

It will also be demonstrated that SFC is not uniformly superior to HPLC. Despite the advantages of supercritical and near-critical fluids in terms of solute diffusion and mobile phase viscosity, there are situations where normal liquids achieve better results.

## EXPERIMENTAL

Two chromatographic systems were used to acquire the data. The first is a modified Gilson HPLC system with a laboratory-made pressure controller that has been described elsewhere [10]. For this study, no column oven was used. The second system was a prototype of the Hewlett-Packard G1205A SFC with two pumps utilized in the downstream mode [11]. Both systems allowed independent pressure and flow control. Detection was performed by high-pressure UV–Vis detectors. The modified Gilson system utilized an IBM PS/2 computer with LABNET (Thermo Separation Products) for data collection while the Hewlett-Packard prototype employed a personal computer-based workstation. Chromatographic conditions are given with the chromatograms. The $^1$H NMR experiments are done as reported in ref. 12.

Carbon dioxide was obtained from Carbagas (Liebefeld, Switzerland; 99.95%) and Scott Specialty Gases (Plumsteadville, PA, USA) and used as received. Liquid modifiers were acquired from Fluka (Buchs, Switzerland) and Burdick & Jackson (Muskegon, MI, USA). Additives and solutes were purchased from Sigma (St. Louis, MO, USA), Merck (Zürich, Switzerland), Fluka or obtained in our laboratory. Chiral columns (Chiralcel OD, OBH and ODH) were either purchased or kindly donated from Daicel, Chiral Technologies (Exton, PA, USA), Stehelin (Basle, Switzerland), or prepared according to the procedure reported by Okamoto *et al.* [13]. The columns were washed in pure methanol at 0.5 ml/min for 30 min before and after use.

## RESULTS AND DISCUSSION

Packed-column SFC can provide a significant reduction in analysis time while maintaining enantiomeric resolution. Fig. 1 illustrates baseline resolution of *R,S*-benzoin. With the same column used in normal-phase HPLC for similar resolution [14], the total analysis time is 21 min ($R_s = 3.33$, $\alpha = 1.23$). As previously stated, this advantage is due to the improved diffusion and viscosity of super- and subcritical fluids which permit higher flow-rates. Figs. 2 and 3 also demonstrate cases where SubFC either provides faster analysis times or resolution that is unattainable with HPLC.

Although the application of SFC or SubFC conditions usually results in an improved resolution over normal-phase HPLC, some solutes are better separated under HPLC conditions. This is the case for phenylalaninol, a primary amine, which is well resolved by HPLC and practically unresolved in SubFC mode (Fig. 4).

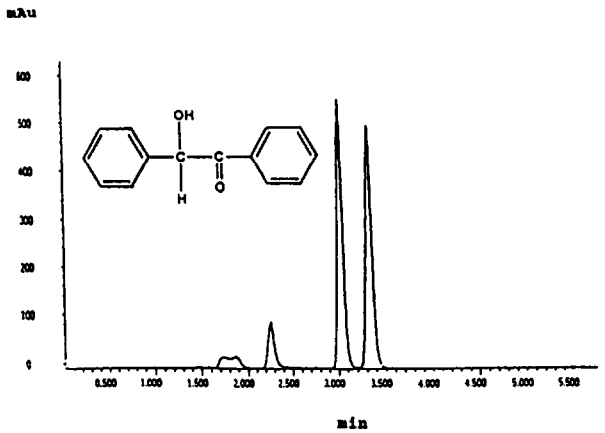


Fig. 1. Chiral resolution of a racemic mixture of benzoin on a Chiralcel OBH (250 × 4.6 mm, 5 μm) column. Conditions: flow-rate 2 ml/min of 20% methanol in carbon dioxide, 150 bar outlet pressure, 35°C oven temperature, 5 μl injected, UV detection at 210 nm, 4 nm bandwidth.

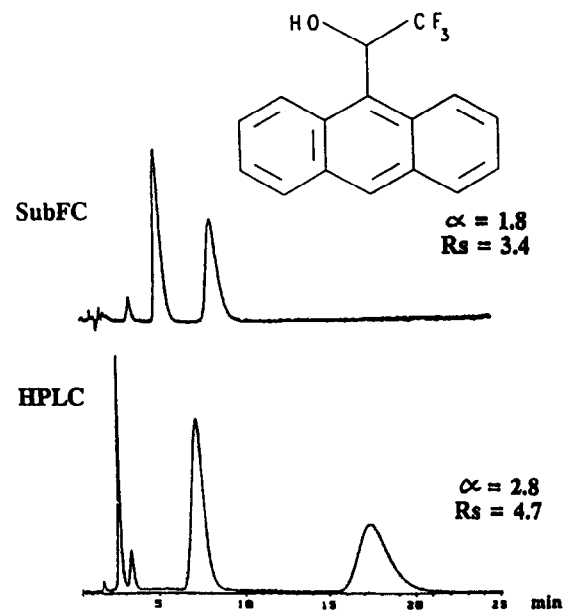


Fig. 2. Comparison of HPLC and SubFC chiral resolution of a racemic mixture of 2,2,2-trifluoro-1-(9-anthryl)-ethanol on a Chiralcel OD column. SubFC conditions: flow-rate 4.5 ml/min of 10% ethanol in carbon dioxide, outlet pressure 225 bar, column at room temperature, 5 μl injected, UV detection at 210 nm. HPLC conditions: flow-rate 1.5 ml/min of hexane–isopropanol (90:10), 10 μl injected, UV detection at 230 nm.

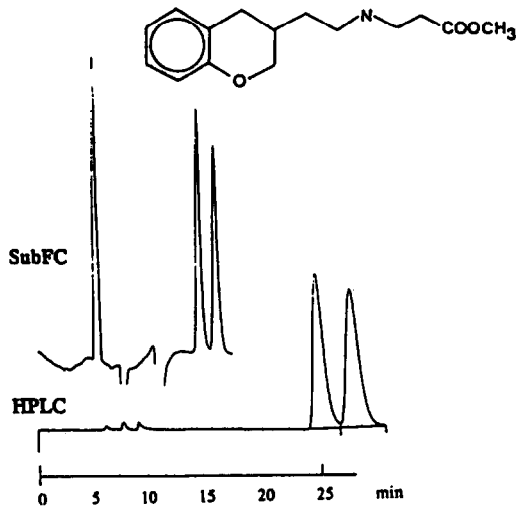


Fig. 3. Comparison of HPLC and SubFC chiral resolution of a racemic mixture of CGP 39540A (N-[2-(dihydrobenzofuran-3-yl)ethyl]-β-alanine methyl ester)·HCl on a Chiralcel OD column. SubFC conditions: flow-rate 2.1 ml/min of 4.8% (methanol with 0.5% ammonium acetate) in carbon dioxide, outlet pressure 225 bar, column at room temperature, 5 ml injected, UV detection at 210 nm. HPLC conditions: flow-rate 0.5 μl/min of hexane–isopropanol (92:8), column at room temperature, 10 μl injection, UV detection at 276 nm.

Similarly, the racemic secondary amine derivative CGP 49823 (substance P antagonist intermediate) is not resolved in the presence of carbon dioxide, while good resolution is obtained on Chiralcel OD under HPLC conditions (Fig. 5).

These results contrast with the findings of Lee *et al.* [8] who investigated a series of β-blockers, a class of compounds with secondary amine functionality, under SFC conditions on the same cellulose-derivative stationary phase (Chiralcel OD). They did not observe such a strong difference between HPLC and SFC selectivity. Siret *et al.* [12] who investigated the same series of racemic drugs observed greater selectivity on a tyrosine-based chiral stationary phase with SFC over HPLC. The authors postulated that the formation of a rigid complex between the carbon dioxide and the solute provided the improved resolution. This explanation was supported by their $^1$H NMR experiments and by molecular modeling investigations. These experiments indicated a change in the electronic density of the

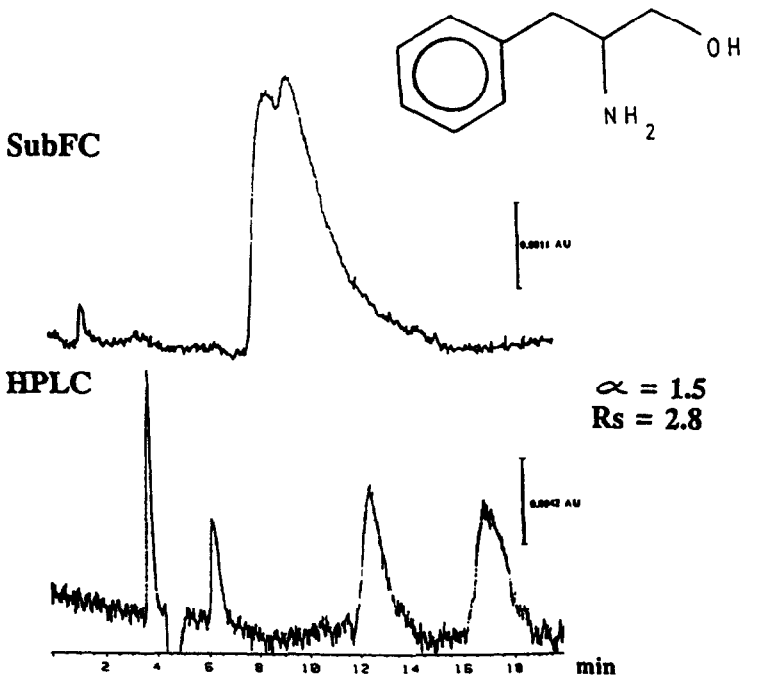


Fig. 4. Comparison of HPLC and SubFC chiral resolution of a racemic mixture of phenylalaninol on a Chiralcel OD column. SubFC conditions: flow-rate 3.2 ml/min of 6.2% isopropanol in carbon dioxide, outlet pressure 225 bar, column at room temperature, 5 μl injected, UV detection at 210 nm. HPLC conditions: flow-rate 0.8 ml/min of hexane–isopropanol–trifluoroacetic acid (97.4:2.5:0.1), column at room temperature, 10 μl injected, UV detection at 215 nm.

nitrogen atom in the molecular conformation in the presence of carbon dioxide.

Under similar experimental $^1$H NMR conditions, chemical shifts were observed for all the aliphatic protons of phenylalaninol to lower field and a change of the coupling constants, especially for the benzylic protons. Such a change in NMR behavior suggests a strong interaction with carbon dioxide. Nevertheless, contrary to the effect seen with β-blockers, this interaction is associated with a decrease in chiral resolution. Unlike phenylalaninol, the other basic compound, CGP 49823, shows no change in its $^1$H NMR spectrum in the presence of carbon dioxide. Although a direct comparison of solute behavior is not feasible due to different stationary phases, the results of the present study and the aforementioned citation indicate that carbon dioxide influences the chiral recognition process. This influence can be beneficial or detrimental.

It is known that ammonia and primary amines interact with carbon dioxide [15–18]. Such an interaction may explain the shifts in $^1$H NMR spectra. The solute complex resulting from the interaction with carbon dioxide can exhibit a different resolution than the original solute. This ability will depend, as in any chiral separation, on the solute structure, mobile phase composition and stationary phase identity. While this effect was positive for the β-blockers on a tyrosine phase, a negative result was obtained for phenylalaninol and CGP 49823 on Chiralcel OD. The NMR studies make it clear that more work in this area is required to find a relationship between NMR data and chromatographic observations.

There appears to be an implicit assumption in the literature that a change from hexane- to carbon dioxide-based mobile phases should only change the physical (as opposed to chemical) characteristics of the fluid. Thus, higher diffusion coefficients and lower viscosities should produce the same resolution faster with lower pressure drops in the carbon dioxide-based fluid. However, both the solute–mobile phase and mobile phase–stationary phase interactions are expected to be substantially different in the different mobile phases. On achiral silica-based stationary

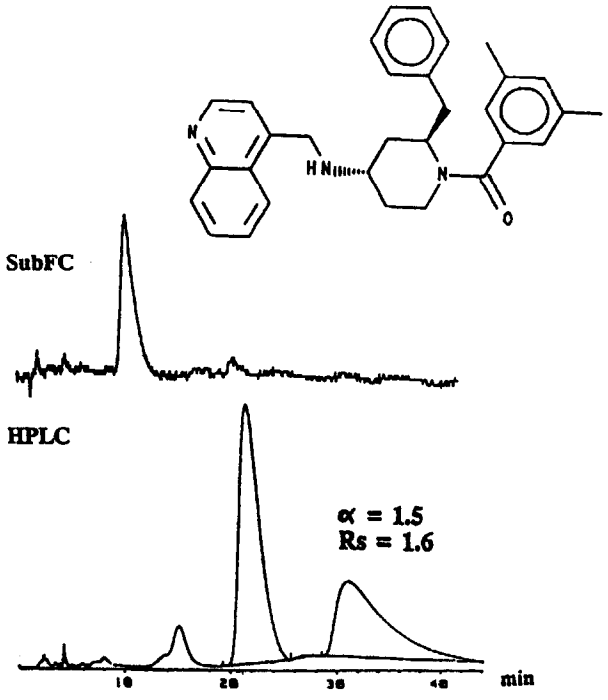


Fig. 5. Comparison of HPLC and SubFC chiral resolution of a racemic mixture of CGP 49823 [(2*R*,4*S*)-2-benzyl-1-(3,5-dimethylbenzoyl)-N-(4-quinoline-methyl)-4-piperidineamine] on a Chiralcel OD column. SubFC conditions: flow-rate 3 ml/min of 3.3% isopropanol in carbon dioxide, outlet pressure 175 bar, column at room temperature, 5 μl injected, UV detection at 248 nm. HPLC conditions: flow-rate 1.5 ml/min of 0.1% diethylamine in hexane–isopropanol (75:25), column at room temperature, 10 μl injected, UV detection at 230 nm.

phases, carbon dioxide and alcohol modifiers are known to undergo greater multilayer adsorption [19,20] than is typical in hexane–alcohol mixtures. Similar behavior should be expected on a chiral stationary phase. Low concentrations of polar modifiers are also known to produce unexpectedly large solvent strength enhancements [21] when added to carbon dioxide. Attributing resolution changes to any one interaction cannot be considered as definitive with the limited data available.

## CONTROL PARAMETERS

With packed-column optimized hardware [10,11], the analyst now has full control over composition, flow, outlet pressure and column temperature. In addition, all or some of these parameters can be programmed to aid in separations. This additional functionality can sometimes make SFC a little overwhelming to the potential user. The effects of these parameters where studied in detail for the tranquilizer hydroxyzine. A ternary mobile phase of carbon dioxide, methanol and isopropylamine was employed to achieve the chromatogram in Fig. 6. In this instance, isopropylamine mainly functions to improve peak shape and does not influence chiral separation [22]. Figs. 7 and 8 illustrate the effect of mobile phase composition and temperature on resolution, respectively. While the effect of composition is mostly due to retention, temperature truly affects the selectivity of the system. Pressure and flow only marginally influence resolution. This is in good agreement with similar studies by Mourier *et al.* [4]. One of the advantages of SFC is the rapid equilibration of the system to new conditions. In these parameter studies, the system was ready to run in under 3 min after changing conditions.

For 3-hydroxymethyldihydrobenzofuran, the influence of modifier type is shown in Fig. 9. At constant composition, isopropanol achieves the greatest resolution of the three alcohol modifiers but requires the longest analysis time. In terms of resolution per unit time, methanol is superior to either ethanol or isopropanol.

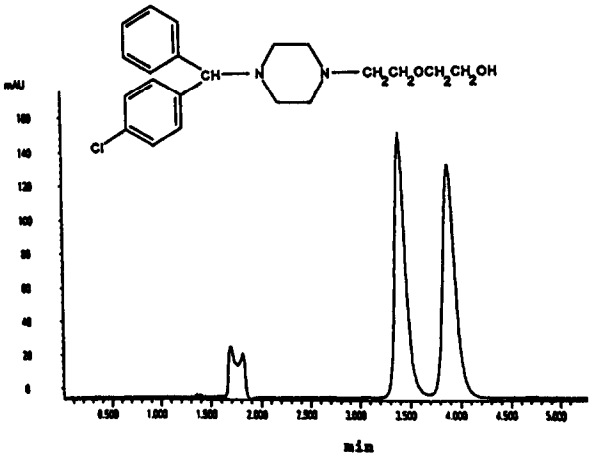


Fig. 6. SubFC chiral resolution of a racemic mixture of hydroxyzine on a Chiralcel OD column. Conditions: flow-rate 2 ml/min of 20% methanol (with 0.5% isopropylamine) in carbon dioxide, outlet pressure 200 bar, oven temperature 35°C, 5 μl injected, UV detection at 220 nm, 4 nm bandwidth.

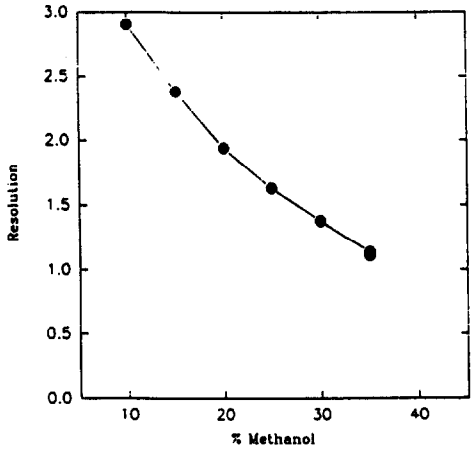


Fig. 7. The effect of modifier concentration in the mobile phase on the chiral resolution of hydroxyzine enantiomers on a Chiralcel OD column. Conditions: flow-rate 2 ml/min, oven temperature 35°C, 150 bar outlet pressure, detection at 220 nm.

## COLUMN STABILITY

One of the concerns with SFC and SubFC is the stability of the columns over long periods of time and many analyses. Fig. 10 illustrates the change in performance for a Chiralcel OD column over 690 h of operation. The operating conditions were 4.5 ml/min of flow at 225 bar with 10% ethanol as the modifier. These parameters exceed the manufacturer's recommended HPLC conditions four-fold in flow and pressure. Furthermore, the pressure at the inlet of the column is easily five times the suggested pressure

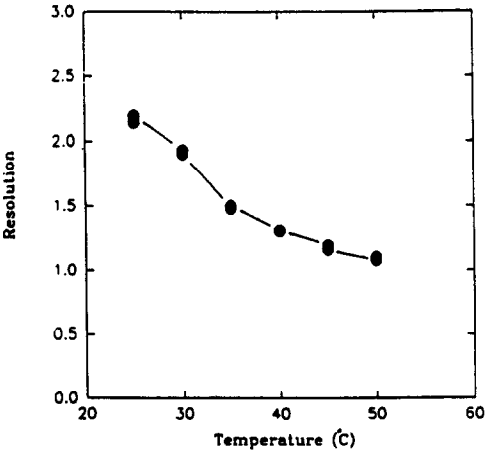


Fig. 8. The effect of oven temperature on the chiral resolution of hydroxyzine enantiomers on a Chiralcel OD column. Conditions: flow-rate 2 ml/min of 20% methanol (with 0.5% of isopropylamine) in carbon dioxide, outlet pressure 150 bar, UV detection at 220 nm.

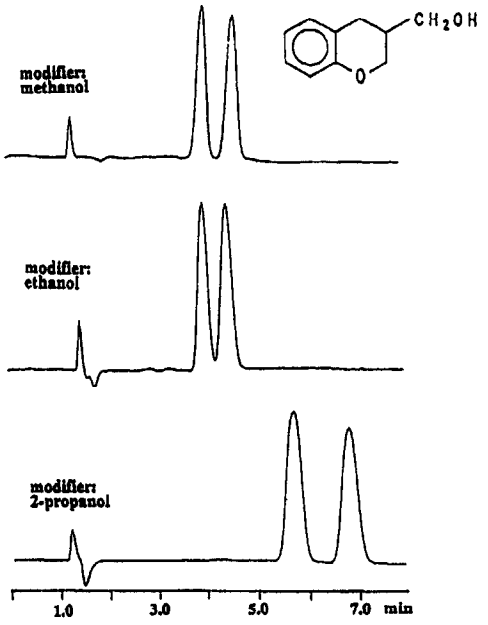


Fig. 9. The effect of modifier identity on retention and resolution for a racemic mixture of 3-hydroxymethyldihydrobenzofuran on a Chiralcel OD column. Conditions: flow-rate 2 ml/min of 2.5% modifier in carbon dioxide, outlet pressure 225 bar, column at room temperature, 5 $\mu$l injected, UV detection at 210 nm.

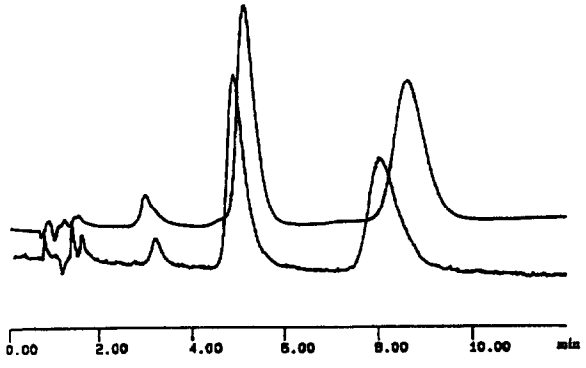


Fig. 10. Long term stability of a 250 × 4.6 mm, 10 $\mu$m Chiralcel OD column. Lower trace: initial separation of 2,2,2-trifluoro-1-(9-anthryl)-ethanol. Upper trace: same column after 690 h of operation. Conditions: 120 l of carbon dioxide and 13 l of ethanol delivered, outlet pressure 225 bar, flow-rate 4.5 ml/min, column at room temperature, UV detection at 210 nm.

limit. Pressure drops of 100 bar have been repeatedly used with these columns without any apparent damage. The decrease in viscosity for super- and near-critical fluids allows this substantial increase in flow-rate while maintaining efficiency. Previous chiral separations of β-blockers [22] were performed at various times over a period of six months. Throughout this time frame, the column never required more than a pure methanol wash to restore its performance. Similar results are being achieved with other cellulose-based columns.

## CONCLUSIONS

Packed-column SFC and SubFC exploit the advantageous viscosity and diffusivity of these fluids for the resolution of chiral compounds. These fluids permit the use of higher flow-rates and can produce higher resolution of enantiomers than HPLC in some cases. Further, the quantity and costs of combustible organic solvents required for such separations can be dramatically reduced.

On the other hand, the superior characteristics of these fluids do not guarantee superior performance to HPLC. In several instances, apparent interaction between basic solutes and carbon dioxide can produce less resolution of the chiral compounds by SFC or SubFC than by HPLC.

## ACKNOWLEDGEMENT

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