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Supercritical Fluid and Unified Chromatography

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Review Contents

Supercritical fluid chromatography (SFC) and related unified chromatography techniques (utilizing compressible, solvating fluids) continue to grow in use. As these techniques reach a new degree of real utility, there is a steady decrease in research dealing with fundamental developments, coupled with a steady rise in the number of industrial users and applications. The overall frequency of papers is down, but this reflects the changing demographics of practitioners from publication-motivated academic researchers to industrial users. Instrument sales are increasing but are still not large enough to gain or sustain the attention of any of the biggest chromatography instrument suppliers.

Nevertheless, progress marches on. Over the last two years, a commercial, dedicated packed-column SFC/MS instrument appeared on the market. Two new commercial column ovens for analytical-scale ultrahigh-temperature HPLC were recently introduced. Both address matching the mobile-phase temperature to the oven before the fluid reaches the column inlet. A commercial split−splitless injector is now available for use with microcolumn SFC. A new 50 mL/min semipreparative SFC instrument appeared commercially from what is now the third supplier of instruments in this size range and bigger. This supplier also offers fraction collection while maintaining enough overpressure to prevent losses due to aerosol formation. Another preparative- and process-scale supercritical fluid extraction supplier is shifting emphasis toward SFC while targeting pharmaceutical customers. Demand from pharmaceutical customers doing preparative SFC has dramatically increased sales of 20-mm-diameter chiral columns. For larger applications, pumping systems are available already in packaged systems that will accommodate 100-mm-diameter columns. Finally, simulated-moving-bed (SMB) separation systems utilizing supercritical fluids have recently been demonstrated. These promise lower eluent consumption and faster production rates than ordinary liquid-based SMB systems.

Among analytical-scale SFC users, the greatest SFC growth has occurred in pharmaceuticals where great speed advances have been reported. Shaving days or weeks off the analysis schedule of a large clinical program is worth millions of dollars in sales and profits if the drug under study is ultimately successful in the market. SFC and reversed-phase- (RP-) high-performance liquid chromatography (HPLC) are complementary and have a large range of overlap in their applicability to pharmaceutical applications. Both techniques encompass about three-quarters of what we could call the application space of pharmaceutical problems. There is a small percentage where only RP-HPLC works. There is a similar percentage where only SFC works. SFC is the obvious choice there, but where the two techniques overlap, SFC-like techniques routinely produce the separations in about one-third the time required by RP-HPLC. Despite the popularity and success record of HPLC for performing separations in pharmaceutical applications, it is very likely that we will soon see SFC become the first choice for high-throughput analysis and purification of combinatorial libraries. The slower and more expensive HPLC will be used as a complementary, secondary technique for the most-polar compounds.

Work similar to SFC but originating from other points of view, such as ultrahigh-temperature HPLC and ultrahigh-pressure HPLC, seems to be more readily received by the traditional HPLC community. Yet, this sort of work reinforces many of the fundamentals embraced by SFC: use of temperature and pressure for mobile-phase strength and selectivity adjustments; and drastic reduction of the mobile-phase viscosity to lower the pressure requirements, increase diffusion rates, and provide much faster flow rates and analysis times. We will cite examples in several of these areas. We will also take a very loose interpretation of "supercritical", for the purposes of selecting articles to cite (and in keeping with the underlying *unified chromatography* theme), to include any solvating fluid that is used in either condensed or supercritical form at a temperature above its normal vaporization temperature or above the usual temperature range of conventional HPLC. Thus, we include very-high- and ultrahigh-temperature liquids, condensed gases used below their critical temperatures, subcritical fluids, supercritical fluids, and solvating gases, with little or no regard to the specific phase state of the fluid.

We resume from our last report (*1*) and have examined the literature abstracted in the *ISI Current Contents Connect* (http://isicc.com/CCC.cgi) database through November 2001. We found about 300 articles from which we have cited a fraction, representative of recent progress, and widely available in libraries worldwide.

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THEORY AND FUNDAMENTAL MEASUREMENTS

Fundamental knowledge of solubility and partitioning behavior is essential in designing chemical processes. Analytical SFC techniques can be used to provide this information. Tuma et al. used an SFC instrument to determine solubilities of a series of anthraquinone dyes and derivatives in several near- and supercritical fluids (*2*). Mishima et al. used SFC methods to determine solubilities of undecanolide and pentadecanolactone (*3*) and flavone and 3-hydroxyflavone in CO_2 (*4*).

Lesellier et al. studied the retention behavior of vegetable oil triglycerides as a function of fatty acid composition and determined the dependence of retention on carbon number and unsaturation (*5*, *6*). Brantly et al., using a novel spectroscopic technique, measured the partitioning of both cosolvents (modifiers) and solutes in CO_2-based systems in contact with polymers (*7*). Yaku et al. compared SFC retention with GC using a McReynolds-like scale and found mobile-phase-dependent differences in the behavior of cyclic versus noncyclic compounds (*8*). Evans and Davis examined pressure effects on retention in reversed-phase liquid chromatography (*9*). They included observations of ionization and complexation changes with pressure and their effects on solute retention.

Solution properties, such as partial molar volumes and enthalpies of transfer, can be derived from SFC retention measurements. Roth et al. applied this to a series of heavy *n*-alkanes and two fullerenes (*10*). Jeon et al. examined partial molar properties of azulene and acenaphthylene (*11*), and naphthalene and biphenyl (*12*) in CO_2, accounting for compositional changes in the stationary phases. This work narrows the gap between the results of chromatographic and nonchromatographic measurements, but the results of these approaches are still not in complete agreement.

Diffusion coefficients are of great interest to designers of extractors and fractionators and can be determined by chromatographic measurements. Funazukuri et al. measured diffusion rates of acetone, phenol, α-tocopherol, and β-carotene in CO_2 (*13*, *14*). Bueno et al. determined diffusion coefficients of benzene and several derivatives in CO_2 (*15*), and Rezaei and Temelli measured diffusion of several free fatty acids and their methyl and ethyl esters (*16*).

MOBILE PHASES AND (ACHIRAL) STATIONARY PHASES

Chromatography, as usually practiced, is a diffusion-limited process. Using smaller particles makes the chromatography go faster because the distances required for diffusive transport are made smaller. Increased pressure is the price of using smaller particles. Clearly, the opportunity of lowering the necessary pressure and simultaneously increasing diffusion rates by elevating the temperature are extremely important in any discussion of ultrafast HPLC. If elevating the temperature results in performance benefits, then there is no reason to stop the progress at the normal mobile-phase boiling temperature if all the solutes and all the system components are still thermally stable—by pressurizing the column outlet, *liquid* mobile phases can continue to be used at superheated temperatures. This is the essence of *unified chromatography* when approached from a starting point of conventional HPLC. There has been considerable recent work in high-temperature HPLC. It is only a matter of semantics to decide where this work ceases being HPLC and enters the realm of this review.

Kirkland discussed the topic of ultrafast reversed-phase HPLC comparing performance of porous and nonporous ultramicroparticles (with diameters of $\leq 2\ \mu$m), monoliths, and superficially porous particles (*17*). He noted problems with pressure requirements as particle sizes are reduced and the benefits of working at elevated temperature to lower the mobile-phase viscosity and improve diffusion rates. However, the attention of this report was clearly on stationary phases and not temperature.

Carr's group at the University of Minnesota has been especially active in high- and ultrahigh-temperature HPLC. Yan et al. investigated heat transfer and its effect on band broadening and the effect of temperature on pressure drop and optimized flow rate in ultrahigh-temperature HPLC (*18*). They separated alkylphenones 50 times faster than conventional HPLC. Thompson et al. looked at the broadening effects caused when the temperatures of the incoming mobile phase and of the column inlet are different (*19*). From another group, Djordjevic et al. examined temperature effects and temperature programming in HPLC and found that heating differences must be reconciled if a method is to be transferred to another instrument with a different heating configuration (*20*).

Temperature effects in liquid chromatography become more pronounced when the temperature approaches or exceeds the normal boiling temperature of the mobile phase and especially if the temperature approaches or exceeds the critical temperature. Mao and Carr described a novel thermally tuned, tandem-column technique in HPLC in which two columns with different selectivities are coupled in sequence with the temperatures of the two columns independently controlled (*21*). Varying the temperature of one column relative to the other causes a marked change in the overall selectivity. This effect is easily controlled and tunable using the temperatures as the primary control parameters.

Fields et al. separated steroids with superheated water in reversed-phase HPLC using zirconia-based stationary phases, noting that superheated water was a suitable replacement for acetonitrile/water mixtures at lower temperatures (*22*). Selectivity was completely different from that with silica-based columns at conventional temperatures with acetonitrile modifier, thereby providing additional choices and flexibility in methods development. Wilson also investigated the separation of several drug compounds using superheated water mobile phase on several different zirconia- and silica-based stationary phases (*23*). Chienthavorn and Smith used inorganic buffers in superheated water to control pH in the reversed-phase separation of sulfonamides (*24*). Separations performed at several different pH values and temperatures allowed an estimate of pK_a values of the sulfonamides as a function of temperature.

HPLC/NMR can be facilitated by using D_2O in place of H_2O in the mobile phase. Signals from modifier components in the mobile phase can be eliminated if the HPLC can be performed without modifier. This, of course, can be done successfully in many cases by heating (or superheating) the D_2O. Smith et al. separated analgesics and caffeine using a polymeric stationary phase and superheated D_2O as the mobile phase (*25*). Column temperatures as high as 190 °C were used with liquid mobile phase, well above normal boiling temperatures. The high temperatures were re-

quired for solute retention control so that organic modifiers could be omitted from the mobile phase for the benefit of the NMR detection. The mobile phase was cooled and depressurized to ambient conditions before detection. The authors noted that, since there is no requirement that the temperature and pressure be kept elevated beyond the column outlet, the NMR measurements could be done at ambient temperature and pressure in the D_2O. When using D_2O, users must be aware of the possibility of deuterium exchange. Smith et al. noted deuterium exchange in methyl groups on a pyrimidine ring when superheated D_2O was used to separate sulfonamides (*26*). The authors also discussed the utility of HPLC/NMR/MS for studying on-column reactions.

Louden et al. used hot and superheated D_2O to separate pharmaceuticals using a reversed-phase column (*27*). They used a variety of detectors including UV, NMR, FT-IR, and MS.

Wu et al. used neat water as the mobile phase in an experiment they described as packed capillary column solvating gas chromatography (SGC) (*28*). The column outlet was not pressurized; however, the mobile phase surely was liquid over much of the column, even at temperatures above 100 °C.

Molander et al. investigated nonaqueous high-temperature HPLC using packed-capillary columns (*29*). Under their conditions, the best efficiency occurred at 100 °C. Anderson et al. reported some very surprising results regarding the retention of poly(ethylene glycol) oligomers as a function of temperature on a reversed-phase system (*30*). They found that retention increased with increasing temperature, the opposite of the expected effect, and that a negative temperature program, from 80 to 25 °C, was required to separate the early-eluting peaks and to elute the high molecular weight peaks.

Zou et al. examined effects of several common modifiers, temperature, and pressure on apparent column efficiency in packed-column SFC (*31*). Modifier-dependent effects on retention and apparent efficiency were reported.

Although a great deal of work has been done to investigate stationary-phase properties for SFC, there is little available for the practitioners of packed-column SFC other than commercial HPLC stationary phases. Ibanez and Senorans reviewed stationary phases for SFC including chiral, liquid crystal, and polymer phases coated on particles (*32*). They also examined tuning the polarity of the stationary phase for optimizing the selectivity of particular separations.

Gritti et al. studied the effects of pressure, temperature, and modifier on the performance of a side-chain liquid crystalline polymer stationary phase (*33*). Various PAH species were used as solutes, and pressure-dependent retention behavior changes were noted.

Wu et al. used packed-capillary-column SFC to separate perfluorinated polyethers and polymethylsiloxane oligomers with CO_2 mobile phase (*34*). Decreasing the particle size and the column length in concert, ultimately to 2 μm and 13 cm, respectively, led to a 7-fold reduction in analysis time while maintaining resolution between the peaks. In other work, Wu et al. compared porous and nonporous, polymer-coated particles in SGC and found that porous particles produced higher peak capacity values for a given mobile-phase velocity (*35*). Wu et al. also compared SGC with open-tubular GC (*36*). They examined several performance aspects such as peak capacity, sample capacity, and pressure drop.

INSTRUMENTATION, TECHNIQUES, AND PERFORMANCE

Thermodynamic measurements in chromatography usually require an accurate knowledge of the column dead volume. Gurdale et al. studied several methods for dead volume determination in subcritical fluid chromatography (SubFC) and concluded that the best method is the use of acetonitrile as an unretained marker (*37*). They classified nine modifiers into four behavior groups according to intermolecular interactions.

There have been several improvements in injection techniques for SFC, but sample introduction is not yet as straightforward as in GC and HPLC. Greibrokk and Chester reviewed SFC injection techniques with emphasis on phase behavior and mass transfer (*38*). Molander et al. described a high-temperature injector for packed-capillary SFC (*39*). Their work was successful in introducing samples, such as synthetic waxes, that were not appreciably soluble in any convenient liquid solvent at room temperature. They also investigated temperature programming. In separate work, Molander et al. used a large-volume injection technique with subambient-temperature focusing to inject up to 100-μL solutions of antioxidant onto 320-μm-diameter packed-capillary columns (*40*). Bruheim et al. coupled temperature-programmed capillary HPLC with FT-IR performing nonaqueous reversed-phase separations of polymer additives (*41*).

The sample solvent is often stronger than the initial mobile phase in SFC methods, and solvent-induced peak broadening places limits on the injection volumes that can be applied. Gao and Kibbey studied the effects of temperature, purge time, purge gas flow rate, precolumn stationary phase, and injection solvent to improve performance of a solvent elimination injection technique (*42*).

Direct connection of a supercritical fluid extractor (SFE) to an SFC instrument for analysis saves a sample-handling step and speeds the analysis. Sato et al. employed this in the determination of capsaicinoids from peppers (*43*). Vanatta et al. described an improved cryo-tee for on-line SFE-SFC (*44*). Brukheim et al. reported how they used a supercritical fluid stream to introduce unstable organometallic compounds into an SFC column for analysis (*45*).

SFC is inherently compatible with virtually every GC and HPLC detector with only few restrictions regarding mobile-phase composition. Most of the possible combinations have now been tried. Herbreteau et al. showed that an unmodified HPLC interface for an evaporative light scattering detector can be used in SFC and produces a 10−30-fold improvement in signal-to-noise ratio for methylated glucoses (*46*). They also successfully interfaced their packed-column SFC to an unmodified atmospheric-pressure chemical ionization mass spectrometer.

PREPARATIVE SEPARATIONS

The needs of people interested in preparative separations may influence the further development and application of analytical-scale SFC, particularly since analytical separations are often used to model and develop preparative separations. Interest seems to be focusing on pharmaceuticals, natural products, and polymers.

Wang et al. used a packed-column, semipreparative SFC system with MS-directed fraction collection for purifying pharmaceutical compounds (*47*). They suggested that this will be "a powerful and complementary technique" to HPLC. Ripka et al. noted that while HPLC can be used effectively to purify libraries containing up to a few thousand compounds, larger libraries are more economically purified using SFC (*48*). They illustrate that over 5000 L of solvent is required to purify a 20 000-compound library by HPLC, but that this is reduced to 120 L with SFC and that 24 days can be saved from the process. Berger et al. developed a semipreparative SFC system capable of pumping at 50 mL/min into 21-mm-i.d. columns and applied it to high-throughput purification of libraries (*49*). They also described a new collection device that prevents losses of materials to aerosols and provides collection efficiencies up to 95% and a "universal" gradient using an amine additive in methanol. Coleman gave guidelines and examples of high-throughput separations using SFC (*50*).

Sasanuma et al. studied polypropylene by C-13 NMR, and fractionated their model compounds by packed-column SFC (*51*). This work was done using a 4.6-mm-i.d. column but was nonetheless a preparative separation. Shimada et al. separated low molecular weight polystyrene oligomers into uniform fractions containing purified solutes of an exact molecular weight (*52*). Hitada, Kitayama, et al. prepared uniform fractions of poly(methyl methacrylate) (PMMA) and hydroxyl-terminated PMMA by SFC (*53*−*55*).

Fish oil preparation is a well-documented example of preparative SFC and has been used in nutriceutical production since the early 1990s. Recently, Alkio et al. examined the economic feasibility of producing concentrates of docosahexaenoic acid and eicosapentaenoic acid (EPA) ethyl esters from esterified tuna oil (*56*). Pettinello et al. developed a preparative SFC procedure to enrich EPA ethyl ester from 68% in the starting mixture to 93% or better (*57*).

Simulated-moving-bed separations can be done using supercritical fluids. Depta et al. described an SMB-SFC plant, which they used for the separation of phytol isomers (*58*). They illustrated the importance of using analytical-scale SFC to first optimize the separation before taking it to the plant. Di Giovanni et al. described an SMB-SFC system used in a pressure-gradient mode (*59*).

More examples are included with Applications, later in this article.

DETECTION

Publications describing advances in detection in SFC have decreased in number compared to previous review periods. This is consistent with the move of SFC from academic and development laboratories into industrial applications. The greatest majority of publications describing detection revolve around "informative" spectroscopic and spectrometric detectors, which provide structural information about eluted analytes.

A prime example of such detection is mass spectrometry (MS). Sjoberg and Markides continued their work in designing atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) probes for open-tubular and capillary column SFC/MS (*60*). They improved the designs of the interface probes and optimized the various factors influencing performance. Detection limits were improved by 20−25-fold in the APCI mode over previous designs and were in the 50 pg−100 fg range. Ventura et al. extended their work in the use of SFC/MS for high-speed screening of pharmaceutically related compounds (*61*). They showed that substituting SFC/MS for HPLC/MS resulted in substantial timesavings and that SFC/MS was compatible with compounds covering a wide range of polarities. Enhanced-fluidity mobile phases also provided improved performance for flow injection analysis. Garzotti et al. demonstrated the advantages of a hybrid quadrupole time-of-flight mass spectrometer for SFC/MS (*62*). Accurate mass measurements enhanced the ability of the system to characterize unknowns in complex mixtures. The high spectral acquisition rate of the system provides better chromatographic fidelity for the narrow SFC peaks. Vela and Caruso reviewed the fundamentals and applications of SFC/inductively coupled plasma-mass spectrometry (ICPMS) for the analysis of organometallic compounds (*63*).

While the technical challenges have been substantial, SFC with nuclear magnetic resonance (NMR) detection may be even more rewarding for mixture characterization than SFC/MS. We mentioned several NMR applications earlier (Mobile Phases and (Achiral) Stationary Phases section) in our discussion of superheated D_2O. Albert included SFC/NMR in a review of NMR coupled to chromatographic separation methods (*64*). Fischer et al. overcame one limitation of SFC/NMR: long spin−lattice relaxation time (*65*). They synthesized and immobilized free radicals within the interface to reduce these relaxation times. In this manner, these researchers obtained integratable ^{1}H NMR spectra with on-line, continuous-flow SFC.

Infrared (IR) spectroscopy is another "informative" spectroscopic detector useful for special applications in SFC. Fourier transform IR spectroscopy detection for open-tubular SFC of the essential oil of hops (*Humulus lupulus*) varieties was studied by Auerbach et al. (*66*). The authors compared spectra of the components deposited on AgCl disks and acquired in an on-line flow cell. The spectra were comparable, but the bands in the flow cell spectra were shifted by 8−10 cm^{-1} to higher wavenumbers than those acquired off-line. Tashiro and Hanesaka used on-line FT-IR detection in the SFC characterization of *n*-paraffins (*67*). The ratios of the C−H and C−C stretch bands were characteristic of the individual paraffins and could be used for identification. The authors also demonstrated quantitative analysis.

Atomic emission spectrometry (AES) is a powerful tool for metal speciation. Coupling SFC with various forms of AES is a great technique for characterizing complex mixtures of organometallic compounds. Vela and Caruso described general considerations in coupling SFC with inductively coupled plasma-based detectors, including AES, as well as applications of SFC/ICP-AES (*63*). In a pair of publications, Bertoncini and colleagues describe the interfacing and applications of SFC/microwave-induced plasma AES (*68*, *69*). Mobile-phase flow rate had little influence on both AES signal and baseline drift (*68*). While the continuous introduction of CO_2 does reduce the available range of emissions, the authors were nevertheless able to study compounds containing P, Cl, N, Si, Fe, and Zn (*69*). Analytes containing N, P, and Zn in automobile lubricants were characterized.

Nitro group-containing explosives were the subjects of a study by Bowerbank et al. (*70*). These researchers used SGC coupled

with chemiluminescence thermal energy analyzer (TEA) detection. This detector is selective and sensitive for nitro functional groups. The power of this instrumentation was demonstrated with the analysis of nitroglycerine in soils.

The evaporative light scattering detector (ELSD) is often used for nonspecific detection of eluates in SFC using modified (i.e., organic solvent-containing) mobile phases. Deschamps and coworkers showed enhanced response for lipophilic analytes with the ELSD by adding triethylamine and an equimolar amount of formic acid to the mobile phase (*71*). This phenomenon was observed for a wide variety of species, ranging from wax esters to ceramides and from phospholipids to sterols. Trones et al. made modifications to a laser light scattering detector allowing its successful use with high-temperature HPLC in a packed-capillary column (*72*).

The flame ionization detector (FID) is one of the most commonly used detectors for open-tubular SFC. Despite its popularity, the work by Wenclawiak and Otterbach is noteworthy (*73*). They discuss the carbon-based calibration of the FID for the determination of pyrethrins. Given that the response of the FID is dependent not only on the carbon content of a molecule but also on the structural arrangement of the carbon atoms, it is not surprising that the authors found that the best calibration standard was allethrin, structurally similar to the pyrethrins.

APPLICATIONS

We have been very selective in the applications included here. We have chosen to only include applications that are new, novel, or otherwise merit mention.

Food-Related Applications. Jochum and Pieper provided an interesting comparison of gas chromatography and SFC in the determination of ω-3-polyunsaturated fatty acids (*74*). When the extracts contain triglycerides, the authors recommend SFC. When bound fatty acids are hydrolyzed and the ethyl esters are formed, the authors recommend either SFC or GC for their determination. Similarly, Lee and Hastilow also compared GC and SFC in the determination of the triacylglycerol profiles of "structured" lipids (i.e., lipids synthesized with medium- or long-chain fatty acids) (*75*). Open-tubular SFC-FID was used. The low temperatures required for this analysis allowed the direct analysis of polyunsaturated structured lipids. In the GC-based analysis, such lipids were hydrogenated to complete saturation prior to analysis. Results from the two methods were in good agreement. This application is noteworthy in the sense that it is a perfect application for open-tubular SFC-FID, a technique that has seen less and less use in recent years.

Zhao and Olesik compared the use of enhanced-fluidity mobile phases (methanol/CO_2 and methanol/CHF_3) to traditional HPLC for the separation of dimeric, trimeric, and higher polymerization products of unsaturated fatty acids (*76*). They found that the SFC-related method provided greater speed of analysis and reduced solvent usage.

Lesellier and his colleagues have been quite active in the development and investigation of SFC and related techniques for the analysis of lipids (*77*−*79*). They took advantage of the low viscosity of the mobile phase to couple five Kromasil columns in series and obtain a high-efficiency separation of ceramides in a reasonable time (*77*). The selectivity of this SubFC separation was quite different from that of a previously developed nonaqueous RP-HPLC separation for saturated and unsaturated ceramides. Lesellier and Tchapla provided a detailed investigation of the influence of various octadecyl columns, the nature and concentration of the modifier, temperature, and pressure on the separation of vegetable oil triglycerides (*78*). The effect of pressure was negligible under the conditions of the separation, but polar modifiers such as acetonitrile and methanol dramatically affected selectivity, and lower temperatures improved selectivity. An optimized separation was achieved with seven coupled Hypersil ODS columns and a mixture of acetonitrile and methanol as modifier. Finally, Lesellier provided an excellent survey of the use of SFC-related methods for the purification and analysis of unsaponifiable lipids (sterols, tocopherols, carotenoids, etc.) (*79*). The influence of pressure, nature and concentration of modifier, stationary phase, and temperature on the separations were described. The use of long, coupled columns was also investigated by Smith et al. for the analysis of fatty acid methyl esters (*80*). Using polar silica columns, the degree of unsaturation had the greatest effect on selectivity.

Fat-soluble vitamins are "naturals" for SFC. Turner et al. described the advantages of both SFE and SFC for this class of compounds (*81*). On-line SFE/SFC is especially attractive for vitamins that are sensitive to light, oxygen, heat, and pH. Senorans and Markides make this point and apply the on-line technique to the extraction and analysis of vitamins from aqueous matrixes (*82*). On-line SFE/SFC was also used by Ibanez et al. for the determination of sensitive tocopherols (vitamin E) for the same reasons (*83*). Silica particles coated with various levels of poly(ethylene glycol) were investigated as a stationary phase. This same group went on to describe the pilot plant-scale SFE of tocopherols from olive pomace, followed by off-line characterization of these species with SFC (*84*). The analysis of more polar vitamins is facilitated by the use of modified CO_2. Pyo used a somewhat uncommon modifier, water, for this purpose (*85*). The level of water in the mobile phase was measured with a novel amperometric microsensor.

The advantages of on-line SFC/SFC were applied to other high-value lipids. Preparative-scale SFC was coupled on-line with SFE for the extraction and fractionation of phospholipids by Taylor et al. (*86*). Soy flakes were defatted using pure CO_2, followed by an extraction with ethanol-modified CO_2 to remove the phospholipids. This extract was deposited on the head of an alumina column, and the phospholipids were fractionated using a stepwise gradient of ethanol/water (9:1) in CO_2. On a much smaller scale, Medvedovici et al. used on-line solid-phase extraction/SFE/SFC for the determination of xanthines (caffeine, theophylline, theobromine) in beverages (*87*). The system was entirely automated.

Fourier transform IR detection was used, both on- and off-line with open-tubular SFC, for the characterization of essential oils from hops (*H. lupulus*) (*66*). Different varieties of hops showed clear differences in the nature and relative ratios of their oil components, as expected.

Natural Products. On-line and off-line SFE/SFC were also favored for many applications to natural product characterization. Ashraf-Khorassani et al. used the off-line combination for the extraction and characterization of kava lactones from kava root (*88*). While pure CO_2 and 15% ethanol in CO_2 provided equal

extraction efficiencies, methanol-modified CO_2 was preferred for the analytical and semipreparative separations. Off-line SFE/SFC was also used by Kohler et al. for the determination of artemisinin in *Artemisia annua L.* (*89*). They used packed-column SFC and ELSD in their work. Bicchi et al. compared SFC/UV and HPLC/UV for the analysis of valerenic acids and valepotriates in *Valeriana officinalis* (*90*). The qualitative and quantitative results for both approaches were comparable, but SFC was faster. The components of cannabis were the subject of the study by Cole using SFC with UV detection (*91*).

Buskov et al. were quite active in the use of SFC and related techniques for the analysis of natural products (*92*−*94*). They studied glucobrassicin and analogues in the Brassicaceae family of plants (*92*). These compounds are postulated to have anticarcinogenic effects. They expanded an analytical method to isolate glucobrassicin and related compounds using preparative-scale SFC. Similarly, they used analytical- and preparative-scale SFC to study sinalbin and related compounds in *Sinapis alba L.*, mustards, and other plants (*93*). They also studied the myrosinase-catalyzed degradation products. Finally, they used the same approach to analyze and purify ascorbigens in Brassica vegetables (*94*). APCI mass spectrometric detection was used by Dost and Davidson to determine atropine in *Atropa belladonna L.* extracts (*95*). Methanol modifier was held at 15% and contained 0.5% trifluoroacetic acid and 0.5% diethylamine as additives.

Bamba et al. studied polyprenols in plants using SFC (*96*, *97*). They found that SFC on an octadecylsilane-packed column provided better selectivities than did HPLC, especially for species containing hydrophobic moieties (*96*). They also found that a phenyl-bonded phase cleanly separated poly-*trans*- and poly-*cis*-prenols (*97*). The presence of poly-*trans*-prenols with degrees of polymerization beyond 9 was confirmed for the first time in plants (*97*). Triterpenoid limonoids, including the natural insecticide azadirachtin, from the seeds of the neem tree (*Azadirachta indica*) were studied by Jarvis and Morgan (*98*). Fractions obtained by solid-phase extraction were separated by SFC. Tavares et al. also studied triterpene-related acids, including betulinic, oleanic, and ursolic acids (*99*). But, in contrast to the work of Jarvis and Morgan, they used open-tubular SFC-FID. Compared to GC, the SFC method had the advantage of not requiring derivatization. While open-tubular SFC is known for long analysis times, Tavares et al.'s separation was completed in less than 15 min with good resolution of the analytes.

Organometallics. The number of applications of SFC to organometallics has significantly grown during this review period. Organometallics are often sensitive to heat, oxygen, and even surfaces and are thus difficult analytes. SFC, especially open-tubular SFC with its low surface area, can be the answer. Organometallic analysis by SFC was reviewed by Wai and Wang (*100*). The authors included recent applications to chelates of transition metals, heavy metals, lanthanides, and actinides and to compounds incorporating Pb, Hg, and Sn.

Bruheim et al. reported on purity testing of organometallic catalysts by packed capillary SFC-FID using pure CO_2 (*101*). Catalysts examined included complexes of W, Ir, Rh, and Mo. This group went further to develop methods to inject and separate air-sensitive organometallics using open-tubular SFC-FID (*45*, *102*). The compounds studied included a variety of complexes of Co and Rh. Samples were solubilized and injected under a pure N_2 atmosphere. No sign of air-induced decomposition was observed. Similarly, Barnes et al. (*103*) and Lin et al. (*104*) used open-tubular SFC-FID for the analysis of zinc dialkyldithiophosphates and of transition metal β-diketonates, respectively. The stability of the β-diketonates of Cu(II) and of Mn(II) was improved by the formation of adducts with tributylphosphine oxide (*104*). In contrast to those using open-tubular SFC for the analysis of organometallics, Geertsen et al. used packed-column SFC for the determination of uranium (*105*). They found that 1-phenyl-3-methyl-4-benzoylpyrazolin-5-one (HPMBP) is a good extracting agent for uranium from a nitric acid aqueous phase and that this agent can be used as a mobile-phase additive to improve the peak shapes of the uranium adducts.

Fossil Fuels, Lubricants, and Synthetic and Natural Waxes. SFC and related techniques have been frequently applied in the area of fossil fuels. The acceptance of these methods by workers in the petroleum industry has grown in recent years. Illustrating this point, Thiebaut and Robert provided a description of the use of SFC methods for simulated distillation and for an ASTM-registered procedure for group-type separations (*106*). They demonstrated improved performance by using multiple detectors and better selectivities using novel stationary and mobile phases. Two other reviews are noteworthy: Barman et al. compare and contrast SFC with other chromatographic methods applied to petroleum and related products (*107*). Rudzinski and Aminabhavi's review is more focused on the use of supercritical fluid technologies (SFE, SFC, and related techniques) for the analytical-scale extraction and characterization of crude oils (*108*). Contrasts with other more conventional methods are also included.

The compatibility of the FID with pure CO_2 mobile phase is a strong asset in the analysis of fossil fuel-derived mixtures. Open-tubular SFC-FID was used by Tavares and Lancas for the characterization of alternative fuels derived from SFE of Brazilian coal (*109*). The inertness of the open-tubular column allowed elution of even polar, higher molecular weight asphaltols. Satou et al. used GC and SFC to generate simulated distribution curves of polyaromatic hydrocarbons in heavy oils (*110*). They also calculated values based upon structure. In results that mirror those of Tavares and Lancas, the authors concluded that SFC was better suited for "nonvolatile" hydrocarbons. Planeta et al. described an interesting application of SFC to the characterization of natural waxes in objects of art (*111*). The sensitive packed-capillary method is an important tool in art restoration efforts.

Oligomers, Polymers, and Polymer Additives. SFC is a powerful technique for the characterization low molecular weight (usually <10 000) polymers. Polysiloxanes are especially amenable to SFC, due to the high solubility of these polymers in liquid and supercritical CO_2. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOFMS) was combined off-line with SFC to determine mass distributions of polysiloxanes by Chmelik et al. (*112*, *113*). They removed the SFC flow restrictor from the FID and inserted it into a vial of acetone to collect fractions at predetermined times for subsequent MALDI analysis. The authors found that distributions determined by the two methods were in good agreement but that slightly higher molecular weight oligomers were detected by MALDI. A similar investigation was conducted by Just et al., but with

water-soluble polysiloxane−poly(ethylene oxide) copolymers (*114*). These authors used SFC-FT-IR and SFC/APCI-MS in addition to MALDI-TOFMS. They found that the two methods incorporating separations were more useful in structure elucidation of both major and minor components, but noted the wide mass range, resolution, and short analysis time of MALDI. They concluded all three methods were complimentary.

Nonionic oligomeric surfactants are well suited to SFC. SFC-FT-IR, SFC-FID, and SFC/APCI-MS were used to characterize SFE extracts from a detergent powder by Auerbach et al. (*115*). These powerful tools were used to identify a variety of surfactants in SFE fractions, most notably ethoxylated alcohols and phenols. Preparative-scale SFC was used by Shimada et al. for the purification of pure polystyrene and ethoxylated oligomers (*116*). To obtain a compromise between the desired peak separation and the maximum analysis time, the authors applied a chromatographic response function, which incorporated four parameters: the initial column temperature, the temperature gradient, the modifier flow rate, and the modifier gradient. In the study of other polymers, Matsunaga et al. developed a high-resolution SFC separation of complex polyacrylic oligomers, such as pentaerythritol-acrylates (*117*). The polymerization of isobutylene was studied by Buchmann et al. using a variety of techniques, including SFC (*118*). The influence of various Lewis acids on the reaction was evaluated.

SFC is also used for the analysis of polymer additives. The combination of on-line SFE with SFC can minimize sample handling and eliminate the use of organic solvents in the analysis of additives, as discussed by Zhou et al. (*119*). The results of on-line SFE/SFC for the extraction and analysis of additives in low-density polyethylene were comparable to those of off-line SFE-HPLC and off-line pressurized solvent extraction-HPLC for all additives except Irganox 1076 (*119*). Audic et al. used SFC to monitor the migration of two additives from plasticized poly(vinyl chloride) flexible-film food wrap into isooctane (*120*). Isooctane was chosen to simulate fatty foods. The experiment was designed to test the effects of various plasma surface modifications and of polymeric plasticizers on the rate of migration.

Achiral Pharmaceuticals. Without doubt, the area of greatest growth in the application of SFC during this review period has been in the pharmaceutical industry. This point was made in reviews written by Anton and Siffrin (*121*) and by Yaku and Morishita (*122*). Anton and Siffrin applied their considerable experience in the area to summarize the aspects of method development related to Good Manufacturing Practices (GMP) and International Conference on Harmonization (ICH) standards (*121*). Standard operating procedures for measuring and calibrating a variety of instrumental parameters such as gradient accuracy, mobile-phase pressure, detector linearity, etc., are described. Illustrative examples provided by Yaku and Morishita include steroids and chiral separations (*122*).

The high speed of SFC separations provides a distinct advantage over conventional HPLC in the high-throughput screening of combinatorial libraries and of synthetic mixtures containing new chemical entities. In these high-speed applications, the effluent is usually directed to a UV absorbance detector, a mass spectrometer, and an ELSD. This type of arrangement was used by Ventura et al., as mentioned earlier (*61*). Berger and Wilson also described high-speed screening of pharmaceuticals (*123*). They explored various mobile-phase additives and demonstrated that SFC can be approximately 5−10 times faster than reversed-phase HPLC for library screening. Regarding speed of analysis, Hoke et al. may hold the current record for the minimum time required to examine a 96-well plate using mass spectrometry and a chromatographic separation for each sample (*124*). They used an eight-injector autosampler, loaded in parallel, and SFC-MS/MS to determine dextromethorphan in plasma extracts. Injection-to-injection cycle time was 5 s. The void time of the system was approximately 1 s, and the retention time of the analyte was approximately 3 s. Accuracy, precision, linearity, and ruggedness were acceptable for sub-ppb analyses.

The fact that the retention mechanism in SFC is usually normal phase but retention is not dramatically influenced by traces of water, as it often is in traditional normal-phase HPLC, can be a great asset in purity screening for pharmaceuticals. As noted by Smith and Berger (*125*), retention and selectivities in SFC differ from those observed in commonly used reversed-phase HPLC screening methods. Thus SFC can reveal impurities not observed by RP-HPLC. The two techniques are complementary. Blackwell et al. took advantage of the normal-phase retention mechanisms and high flow rates of SFC to generate high-efficiency impurity profiles in far less time than required for RP-HPLC (*126*). Roston et al. used the low viscosities of SFC mobile phases not only to increase flow rates but also to extend column length for impurity profiling (*127*). The extended column lengths provided higher chromatographic efficiencies and greater resolution of impurities.

Many publications dealt with the development of SFC methods for individual or classes of pharmaceuticals. Long-time workers in the field, Gyllenhaal and colleagues, described an SFC method for clevidipine, a dihydropyridine pharmaceutical (*128*). The method showed better selectivity than an earlier RP-HPLC method. Gyllenhaal and Karlsson also developed an interesting method for isosorbide-5-mononitrate and impurities (*129*). The separation was conducted on porous graphitized carbon with methanol-modified CO_2. Tetraalkylammonium hydrogen sulfates were added to the methanol to allow elution and determination of free nitrate in the drug. The authors report an almost linear increase in the retention time of nitrate with the carbon content of the quaternary ammonium ion additive.

Patil et al. (*130*), Bhoir et al. (*131*), and Dhorda et al. (*132*) have been very productive in developing SFC methods for pharmaceuticals over the past few years. They published SFC-based assays for tolnaftate and impurities (*130*), for sufadoxine (*131*), a sulfonamide drug, and for actives in over-the-counter analgesics (*132*), respectively. The tolnaftate method was for synthetic mixtures (*130*). While all relevant peaks eluted from a Hypersil C-18 column with pure CO_2 mobile phase, peak shapes were improved by the use of 2% methanol. The sulfadoxine SFC/UV method (*131*) was for the determination of levels of the drug in human plasma extracts. The SFC method was comparable to the published HPLC/UV method in all aspects but speed and use of organic solvents, in which the SFC method was superior.

Sufonamide antibiotics were also the subjects of a study of Dost et al. (*133*). Dost and colleagues developed an SFC/UV/APCI-MS method for the separation and quantitation of six sulfonamides used to prevent infections in livestock. The method was acceptable

in linearity, accuracy, precision, and all other aspects. The authors used it for the determination of sulfonamides at sub-ppb levels in milk. Salbutamol sulfate and its impurities were determined using SFC by del Nozal et al. (*134*).

Antibiotics intended for livestock were also the subject of a study by Ashraf-Khorassani et al. (*135*). The authors developed an SFC method to monitor the level of alexomycin in SFE and pressurized-fluid extracts from animal feeds. They found that the SFC method had better selectivity for alexomycin than an HPLC method and was thereby better suited for quantitative analysis. Toribo et al. developed a rapid method for the determination of diastereomers of 2-bromomethyl-2-(2,4-dichlorophenyl-1,3-dioxolan-4-yl)methyl benzoate (*136*). After investigating a variety of stationary phases, modifiers, and chromatographic conditions, the authors achieved baseline separation of the two stereoisomers with a 2-min cycle time.

Due to their polarity, pharmaceuticals and biologically active compounds are rarely separated with pure CO_2 mobile phase in SFC, and the FID is therefore rarely used. The use of the FID was therefore one notable aspect of the work by Graves et al. in the separation and purification of a labile digitalis-like factor (*137*). These authors used two dimensions of SFC to purify the unknown and used the FID to estimate its amount.

While most SFC-like separations employ a normal-phase retention mechanism, Zhao and Olesik used enhanced-fluidity liquid chromatography with a reversed-phase retention mechanism to separate basic tricyclic antidepressants (*138*). Fluoroform was chosen as the viscosity-enhancing agent and was added to a methanol/phosphate buffer mobile phase. The enhanced-fluidity mobile phase provided decreased analysis time, increased efficiency, lower pressure drop, and improved selectivity. Thompson et al. used high-temperature HPLC to separate amyloid-β-peptide variants, components of the amyloid deposits in Alzheimer's disease (*139*).

Chiral Separations. Chiral HPLC has been most commonly performed in normal-phase mode. The clear advantages of SFC over traditional normal-phase HPLC have propelled SFC into a leadership position in chiral chromatography. Recent reviews by Phinney (*140*) and Terfloth (*141*) are good starting places for someone new to the field or looking for examples of benefits.

Svensson et al. compared enantioselectivity of a variety of acidic and basic drugs using polar organic-phase HPLC, reversed-phase HPLC, normal-phase HPLC, and SFC, all with a vancomycin-coated stationary phase (*142*). They found that bases were separated best using the polar organic mobile phases. Acids worked best with reversed-phase conditions. SFC had the broadest overall enantioselectivity. Normal-phase HPLC was similar to SFC except, under the conditions of this work, normal-phase HPLC produced lower column efficiency than did SFC.

Toribio et al. developed a strategy for chiral separations of dioxalene derivatives and cyclic ketones (*143*). They studied temperature, pressure, and modifier effects; adopted 200 bar, 35 °C, 2 mL/min, and a gradient starting with 5% methanol (with 0.1% triethylamine and 0.1% trifluoroacetic acid) in CO_2 as the mobile phase; and showed this to be applicable to several chiral stationary phases.

Thienpont et al. studied the separation of ketoconazole and itraconazole enantiomers and reported advantages of SFC over HPLC (*144*). Ketoconazole enantiomers were separated with a resolution of 4.29 in less than 7 min by Bernal et al. on an amylose-based column (*145*). Drastic changes in resolution were obtained for different modifiers, with ethanol (modified with triethylamine and trifluoroacetic acid) at 30% providing the best results. Toribio et al. studied the effects of modifier and pressure on the separation of ketoconazole and several other solutes using Chiralpak AD and Chiracel OD stationary phases (*146*). Methanol worked better than acetonitrile as modifier for the examples in this study. Salvador et al. used dimethylated β-cyclodextrins adsorbed on graphitic carbon as stationary phase and found this approach to be very flexible (*147*). They also studied effects of the chiral selector concentration, modifier choice, pressure, temperature, etc., on the separation performance (*148*).

Wu et al. used near-critical CO_2 as mobile phase to accomplish several chiral separation applications on packed-capillary columns in 1 min or less (*149*).

Shen et al. examined the effect of silica pore size on linear velocity, retention, selectivity, efficiency, resolution, and resolution per unit time using an immobilized β-cyclodextrin−polysiloxane coating (*150*). Ellwanger et al. used molecularly imprinted polymers as chiral stationary phases (*151*). They reported several interesting performance effects, depending on the method of stationary-phase synthesis.

Shurig and Fluck described complexation SFC for the separation of Lewis bases on Chirasil-Nickel and Chirasil-Zinc (*152*). They studied effects of temperature, pressure, and density of CO_2 mobile phase on retention and chiral selectivity. Gyllenhaal and Karlsson separated substituted dihydropyridines on Hypercarb using Tween 60 as a deactivation additive and (*Z*)-(L)-arginine as counterion (*153*).

Bernal et al. studied the separation of 1,3-dioxolane derivatives and looked at the influence of modifier, temperature, and pressure (*154*). They found that the best modifier was different for each compound they examined. Svensson et al. separated metoprolol and analogues with Chiracel OD and Chiralpak AD (*155*). They used a chemometrics-based experimental design and found Chiracel OD to be superior, giving separation factors up to 4.5. Temperature was more important than methanol content in their work. (Temperature effects are often much larger in SFC than in HPLC, even when using the same stationary phase, and offer a powerful means of adjusting the separation.) Svensson and Owens compared a commercial ristocetrin A chiral stationary phase with one they prepared themselves and separated enantiomers of dichlorprop, ketoprofen, warfarin, coumachlor, and thalidomide (*156*). Dichlorprop and ketoprofen were separated on the home-made stationary phase but not on the commercial one. Hoke et al. compared SFC with HPLC for the separation of ketoprofen enantiomers in human plasma following 96-well solid-phase extraction (*157*). Their optimized SFC separation method took 2.3 min and was comparable to an HPLC separation that could not be done in less than 6.5 min. Analytical attributes for the two methods were otherwise similar. Johannsen studied the separation of ibuprofen enantiomers on 11 different stationary phases (*158*) and found that Kromasil CHI-TBB was the best in this case. The type of modifier and its concentration had a much stronger effect on the outcome than either temperature or pressure. Ashraf-Khorassani et al. developed and validated an assay for xemilofiban

that provided quantification, purity, and on-line FT-IR identification in less than 30 min (*159*). Precision and accuracy were similar with HPLC−UV assays of comparable analytes. Desmet et al. used Chiralpak AD to separate 2-oxatetracycloundec-9-ene derivatives (*160*). Duval et al. compared SFC and HPLC and found that SFC was superior for separating aminoglutethimide but not thalidomide (*161*). Gyllenhaal was able to complete 10 separations of samples containing clevidipine enantiomers in 5 min using SFC (*162*). He reported a relative standard deviation of 0.6% in the area ratio for a set of 10 replicates.

Stahl et al. used SFC in the study of the possible biosynthesis of a leading HIV protease inhibitor, Indinavir (*163*). In a scaled-up biosynthesis of an important precursor, the authors measured enantiomeric excess using SFC. Many basic pharmaceuticals are present as hydrochlorides. Yaku et al. used SFC for the direct determination of the enantiomers of diltiazem hydrochloride (*164*). Savi et al. used chiral SFC in their study of clopidogrel, which inhibits platelet aggregation (*165*). The authors demonstrated that liver metabolism of the parent drug is necessary for biological activity and that this metabolite can be generated from human liver microsomes.

Miscellaneous Applications. The application of SFC in the forensic sciences is relatively new. Radcliffe et al. described the advantages of SFC and related techniques in this area (*166*). The most common application was in the separation of drugs of abuse, both for time-of-death measurements and for obtaining information related to long-term drug abuse. The authors also described the use of SFC in the characterization of explosives from gunshot residues and bombings. Nitroaromatics used in the production of munitions and explosives were the subjects of a study by Smith et al. (*167*). These authors explored SGC for the rapid determination of energetic materials in environmental samples. In a subsequent paper, Bowerbank et al. continued this study of energetic materials (*70*). They combined SGC with chemiluminescence thermal energy analyzer detection for the selective and sensitive detection of nitro-containing molecules.

Methylated β-cyclodextrins are used for a wide variety of commercial applications. Salvador et al. used a variety of methods, including SFC with ELSD detection, to characterize five commercial dimethylated β-cyclodextrins (*168*). The authors explored a variety of stationary and mobile phases and found that silica and nitro-bonded silica, used with CO_2/methanol/acetonitrile and CO_2/methanol mobile phases, respectively, were most useful.

The speed of SFC is advantageous for the analysis of pesticides and agrochemicals. Dost et al. describe the combination of SFC with APCI-MS for the determination of six pesticide residues in soils (*169*). Detection limits were in the hundreds-of-picograms to low-nanogram range, and soil matrixes provided no interference. Medvedovici et al. developed an on-line solid-phase extraction-SFC/UV method for the determination of carbaryl in water samples (*170*). The method reduced sample handling and achieved a detection limit of 5 ppb with a cycle time of ∼35 min.

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