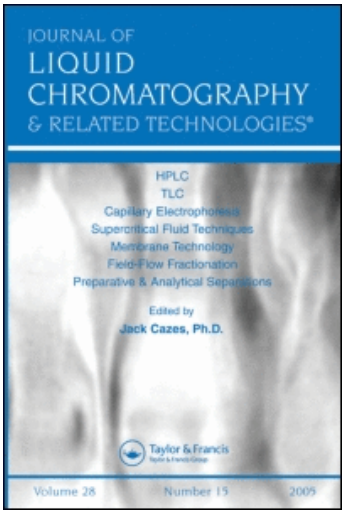

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Advances in High Throughput Supercritical Fluid Chromatography

William P. Farrell[a]; Christine M. Aurigemma[a]; David F. Masters-Moore[a]
[a] Pfizer Global Research and Development, La Jolla Laboratories, San Diego, California, USA

To cite this Article Farrell, William P. , Aurigemma, Christine M. and Masters-Moore, David F.(2009) 'Advances in High Throughput Supercritical Fluid Chromatography', Journal of Liquid Chromatography & Related Technologies, 32: 11, 1689 — 1710

To link to this Article: DOI: 10.1080/10826070902956394

URL: http://dx.doi.org/10.1080/10826070902956394

Journal of Liquid Chromatography & Related Technologies®, 32: 1689–1710, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070902956394

Advances in High Throughput Supercritical Fluid Chromatography

William P. Farrell, Christine M. Aurigemma, and David F. Masters-Moore

Pfizer Global Research and Development, La Jolla Laboratories, San Diego, California, USA

Abstract: Supercritical fluid chromatography (SFC) has long been touted as the separation of choice for high throughput applications and, after nearly a decade, is finally considered mainstream in the analytical chemistry community. Applications range from microscale analysis of complex mixtures to macroscale purification of chiral enantiomers in a variety of industries, such as pharmaceuticals, foods, cosmetics, agrochemicals, petrochemical and natural products. The inherent speed, efficiency, and versatility of SFC have transformed the perceptions of the technology from novelty to integral tool for the modern analytical lab, especially those labs wanting to maximize throughput. However, maneuvering SFC through the transition from single samples to array-like libraries has strained the development of instrumentation to meet the challenging demands. This review presents the major developments and applications useful to those embarking on using SFC for high throughput applications in other fields.

Keywords: Bioanalytical, Chiral purification, High performance liquid chromatography (HPLC), High throughput, Proteomics, Supercritical fluid chromatography (SFC)

Correspondence: William P. Farrell, Pfizer Global Research and Development, La Jolla Laboratories, 10777 Science Center Drive, San Diego, CA 92121, USA. E-mail: bill.farrell@pfizer.com

INTRODUCTION

Supercritical fluid chromatography (SFC) has emerged from relative obscurity in the early 1990's to become one of the fastest forms of high resolution chromatography commercially available today, due to its speed, cost efficiency, environmental benefits, and comprehensive separation capabilities. SFC employs a compressible gas, e.g., carbon dioxide (CO_2), maintained above its critical temperature (T_c) and pressure (P_c), which is often mixed with a miscible organic solvent (e.g., methanol). The technique can be used in conjunction with normal phase or reverse phase stationary phases, but is most commonly used as a replacement for normal phase high performance liquid chromatography (NP-HPLC). This characteristic allows SFC to achieve near chromatographic universality, except in cases of very polar compounds, e.g., inorganic ions and proteins.[1–3] In addition, the SFC mobile phase, in either the subcritical or supercritical state, exhibits lower viscosity and higher diffusivity than normal liquids, thus allowing for higher flow rates at lower column pressure. This translates to faster analyses on longer columns without requiring ultrahigh pressure instrumentation.[2,4] Supercritical fluid chromatography is an environmentally friendly alternative to HPLC because it uses less organic solvent. Since the bulk of the SFC mobile phase is a compressed gas, significantly less liquid waste is produced as a result. Carbon dioxide by itself may not readily be considered environmentally friendly or "green" given its contribution to global warming. However, there are several factors that make CO_2 more attractive than traditional solvents for chemical processes: CO_2 gas is inert, non-flammable, and inexpensive. It is easily and safely liquefied, solidified, handled, and stored.[5] Carbon dioxide is also a component of the atmosphere and does not require combustion to oxidize it to a safe chemical form prior to being released. In contrast, the combustion of fossil fuels and solvents, such as those used in HPLC, introduces harmful byproducts (e.g., CO, NO_x, particulates, etc.) into the environment. Most commercial CO_2 is reclaimed from other industrial processes, such as ethanol, coke, ammonia, and natural gas production, as well as the burning of fossil fuels for the generation of electricity. Each of these processes contributes millions of tons of carbon dioxide each year in the U.S. alone.[6] Since the carbon dioxide used for SFC is already an industrial byproduct, the net environmental impact is zero.[7]

SFC can be operated with both non-polar and polar phases, allowing flexibility by changing the stationary phase polarity or by coupling columns of differing polarities together.[8,9] Several groups have reported that SFC column lifetimes were higher relative to HPLC, which is an especially important consideration for chiral chromatography due to the significant expense of chiral columns.[10–12] Chiral chromatography

is probably the most successfully demonstrated use of SFC. Over the last decade, SFC has become the technique of choice for analysis[12–27] and purification[28–35] of enantiomers. As the technology matures, it could eclipse HPLC for analysis and purification of compounds ranging from mg to kg scale. In addition to chiral separations, SFC has been used in numerous applications including pesticides,[36–42] natural products,[43,44] petrochemicals,[45] and pharmaceuticals.[26,34,46–55] Its wide applicability and demonstrated analytical and preparative benefits have helped define SFC as the new high speed paradigm in analytical chemistry. While the topic of high throughput (HT) SFC analysis and purification has been covered in several additional reviews,[24,52,56–61] the focus of this review will discuss the factors to consider before contemplating a HT SFC application, advances in stationary phases, and those emerging applications that have the potential to expand the use of SFC into the future.

High Throughput Considerations

The success of any HT analytical or preparative operation is contingent on the development of the detailed workflow to achieve the desired outcome in an expeditious manner. When defining the desired throughput, more than just the daily capacity of the chromatographic system should be considered. Repetitive tasks such as sample filtration, plate sealing, and vial crimping should be examined because they are important components of the overall workflow that can add unintentional delays. How do these devices or tasks fit within the entire workflow? Can they be automated, consolidated, or optimized to gain efficiency? At higher throughput, human manipulation must be minimized, as the number of samples per unit time increases beyond what is possible for a person to complete within a reasonable timeframe. By considering the process in its entirety, the overall value of the effort can be maximized and the need for manual resources is reduced, enabling the most efficient decision making, product selection, and overall time savings. With automation, the ability to review and store electronic data related to each process step must be enabled. Additionally, instrumentation should be capable of unattended operation for extended periods of time.

Electronic Data

Obtaining and utilizing as much information about the samples upon submission from one group to another (e.g., synthesis to purification, or synthesis to screening, etc.) is critical to the success of the HT process. Data inputs and outputs are defined and optimized for the particular workflow, physical sample characteristics, and instrumentation requirements.

In order to manage the flow of data within a workflow, customized software is usually required. This software should be capable of tracking the various workflow stages: submission, fractions, detector, and chromatography reports, and should be linked to either relational databases or electronic notebooks for data extraction and utilization. A representative diagram is presented in Figure 1. Instrument work lists are generally tabular in design and can easily be generated from the database and electronically downloaded to the instrument control software to avoid errors and minimize manual intervention. Acquired data is automatically processed and tabular results can be uploaded to the repository database, where archived data can be later retrieved, reviewed, and scrutinized. Hochlowski et al. described a process for chemical library analysis and purification that utilized custom software to extract large data sets from a chemistry electronic notebook (ELNB), labeling and tracking samples through various steps within the process and generating chromatographic reports, which were again stored in the ELNB. The process had a reported capacity of 25,000 samples per year with a 72 hour turnaround at 10–300 mg per sample.[58]

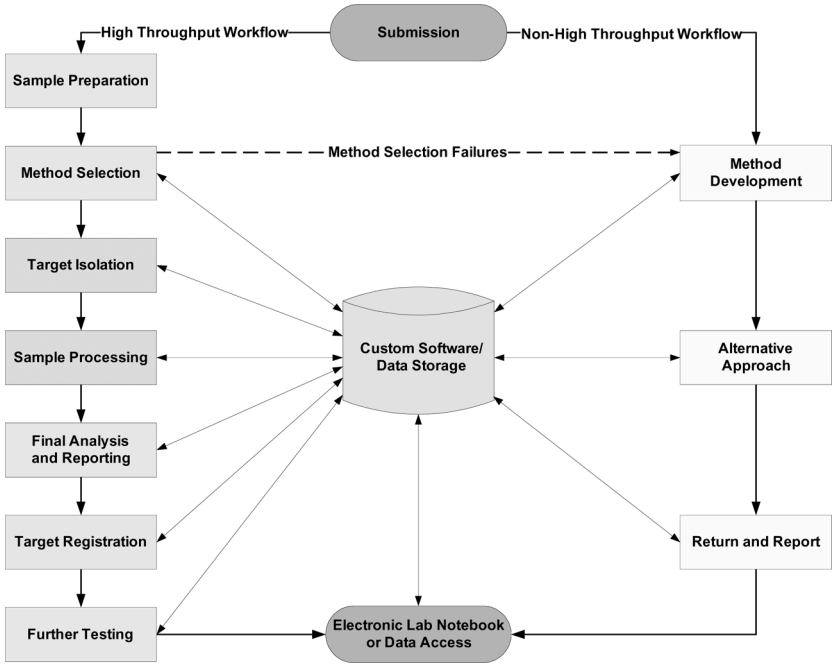

Figure 1. Diagram of a high throughput process workflow. The arrows represent the flow of data and/or samples. Cross-shaded areas represent optional purification workflow.

For HT purification, in addition to any data needed for identification and tracking of samples, the sample solubility, desired purity, and scale all should match the chromatographic system. Purification process steps are the same whether using SFC or HPLC and include sample submission, analysis, isolation, data reporting, and sample registration. High throughput demands dictate which sample format is used: 96-well, 384-well, and 1536-well plate formats are the most common in HT analytical and purification operations, and these are tracked with barcodes associated with an appropriate database. The purification of new chemical entities from a synthetic reaction mixture removes the target molecule from its impurities. Ideally, only one target compound is purified into a single fraction tube, which simplifies the process requirements. For most purifications, this is relatively straightforward; however, isomeric reaction mixtures can complicate the separation, identification, and fraction tracking of target molecules, especially when mass spectrometric detection indicates that the chromatographic peaks have the same molecular ion. As synthetic reactions are not always predictable, it is possible for target impurities to also be isomeric and, therefore, the purification process must be designed to handle isomeric targets, multiple fractions, and multiple fraction analyses assigned to each fraction when necessary. The target product of an isomeric purification is not considered pure until structural confirmation of a single isomer in one tube is obtained, with supporting data assigned to the correct sample identifier. Custom software and hardware must be capable of dealing with such complex situations.

Instrumentation Requirements

The use of commercial instrument hardware and software enables efficient sample data transfers between database and instrument for control and reporting. Robust instrument systems that can process hundreds of samples per day with minimal instrument or software downtime are the benchmark of a well designed high throughput process. Also, the ability for unattended operation is a key consideration for maintaining productivity when comparing commercially available systems.

To facilitate data transfer and reduce the complexity of software and hardware components from multiple vendors, a chromatographic instrument platform from a single vendor is preferred. Not only does this simplify the integration of hardware components with a single operating software or custom software platform, it reduces the need for additional user training, reduces the need for parts from multiple vendors, and increases the ability to swap components. Choosing a system that offers flexibility to customize the instrument with additional features without changing the hardware/software interface usually requires working with a single vendor.

Every facet of the HT process, whether it is hardware or software, has the potential to malfunction. Individual process steps relying on single instrument systems are at risk for disrupting the entire process. Duplicate instrument systems operating in parallel at each process step prevent such disruptions, as well as increase throughput capacity. Database backup and workstation imaging ensure a timely method of restoring software capabilities. Although a fully integrated single instrument or software approach seems beneficial, severe disruptions occur when the instrument stops or malfunctions, causing potentially lengthy process interruptions and downtime.[62–64] Having redundancy within the system or duplication of instruments allows for facile rerouting of samples to maintain continuity and throughput without sacrificing data quality. For instance, utilization of centralized database servers, which connect multiple sites within an organization, is a risk; when the server fails, all sites are adversely affected. However, the use of localized data storage does not affect other functioning sites if one server malfunctions. Although this can drive up maintenance costs, calculating the return on investment is easily performed. Therefore, high throughput process success is dependent on hardware and software redundancy, backups, and failure planning, and ensuring process capabilities are available at all times. Establishing the infrastructure, procurement of equipment, solvent supply, and waste disposal requires planning. Additionally, securing the required space that could potentially expand as the operation grows are factors which also warrants consideration.

Advances in SFC Stationary Phases

SFC is generally considered to be similar to normal phase chromatography, although the exact mechanism stills seems to be the subject of study and even suggests that it can work with both polar and non-polar stationary phases.[2,3,8,65,66] Mass spectrometry (MS) detection is typically used for HT operations, along with columns packed with polar, basic ligands such as 2-ethylpyridine or morpholine, which are usually employed. These stationary phases provide diverse separations, compatibility with acidic/basic solutes, and can be used without counter ions that could diminish ionization.[61,67,68] Gradient elution is almost always necessary to achieve both separation and elution of the solutes.[58–61,68,69] However, if an adequate separation is not achieved, changing the stationary phase has the greatest effect on the separation.[2,70] Adding miscible counter ions such as ammonium formate or ammonium acetate have been shown to improve peak shapes.[49,71,72]

Mixed phases or coupled columns can also be employed to enhance the selectivity of separations in HT mode by reducing the need to

interrupt the process and switch to an alternative column.[67] If column switching is employed to refine method development, it appears that an assortment of column types such as pyridine-type phase (2- or 4-ethylpyridine or mixed mode), diol, morpholine and cyanopropyl for polar compounds, are suitable alternatives.[49,61,67,68] For non-polar compounds, an array of cyanopropyl, C18, phenyl, and pentylfluorophenyl (PFP) columns should be evaluated.[8,57,72,73] Additional columns that could be utilized, depending on the specific application, include silica, testosterone, and porous graphite carbon phases.[66,67,74,75]

If mass spectrometric detection is not used, the addition of acidic or basic counter ions will dramatically improve peak shapes and decrease retention from polar stationary phases. SFC has also been successfully used with ion pair reagents. The β-adrenoreceptor blocking agent metoprolol and its related amino alcohols were separated by using a diol column and a series of ion pairing reagents. Gyllenhaal, et al. examined the use of triethylamine (TEA) with trifluoroacetic, ethanesulfonic, heptafluorobutyric, tridecafluorooctanoic, and octanesulfonic acids, for separations using a Lichrosorb diol column. They found using standard conditions of 10% methanol with 24 mM of the acid and 18 mM of TEA that retention was affected by the structure of both the acid and the solute.[76] Varying the lipophilicity and concentration of acid indicated that selectivity and retention are not dictated by the counter ion alone. Zheng and Brunelli both reported similar chromatographic improvements when using ammonium acetate or ammonium formate counter ion pairs.[71,72,77,78]

If retention is not achieved using polar columns, switching to non-polar columns can be warranted. West et al. have published detailed works on the use of alkyl phases for exquisite separations of steroids, non-steroidal anti-inflammatory drugs (NSAIDs), xanthines, purines, pyrimidines, sulfonamides[1,49,67,72] sunscreens,[70] alkylbenzenes,[1,65,66] carotenoids,[1] triglycerides,[1] lipids, ceramides, phospholipids, and glycolipids,[67,72,79] that could potentially be optimized for HT applications.

HT Applications

SFC technology has been applied to a number of high throughput analytical and purification functions stemming from the increased demand for higher throughput while maintaining data quality without increasing operational or solvent expenses. Many of these applications have emerged from the need to increase throughput in drug discovery efforts in the pharmaceutical industry, and presented as examples of applied high throughput SFC technology.

SFC/MS Applications

Ventura et al. compared high throughput packed column SFC/MS to LC/MS analysis of combinatorial libraries using a modified SFC/MS system consisting of an Agilent 1100 LC/MSD with diode array detection, a Berger SFC, and a Gilson 215 liquid handler.[61] The system was highly customized as each component was controlled by either the Agilent or Berger operating software on two separate computers, and synchronized by custom software. Highly diverse combinatorial libraries were analyzed with negative mode atmospheric pressure chemical ionization (APCI) detection using a diol column and gradient elution of methanol containing 0.3% isopropylamine and 1% 1,2-dichloroethane additives, with a cycle time of 3.5 minutes between injections. A thiohydantoin combinatorial library analyzed by both LC/MS and SFC/MS showed that SFC had superior resolution with 60% shorter cycle times. The authors claimed high throughput SFC analysis of over 400 samples every 24 hours without user intervention. Ventura et al. later removed the additives from the mobile phase modifier to enable positive mode APCI ionization. The gradient profile and flow rates were optimized to improve impurity separation and an auxiliary solvent flow to the MS stream was introduced to enhance sensitivity and detection for diverse combinatorial libraries.[68] A robust, practical high throughput SFC/MS analytical method using only CO_2/methanol was then derived, which outperformed their standard LC/MS methods. Using this method, the authors obtained a throughput of up to 1000 samples per 24 hours with unattended operation.

While SFC/MS with APCI is more commonly utilized, successful electrospray ionization (ESI) with the SFC/MS interface has been reported.[80,81] In one study, the absence of high voltage during ES ionization in SFC/MS has been shown to unexpectedly increase the signal intensity for a group of standard samples. Thite et al.[82] tested the effect of different post-column makeup fluid compositions, as well as the effects of pressure, SFC flow rate, and modifier composition on the signal sensitivity under high voltage and zero voltage MS conditions for both ESI and APCI sources. Reference standard signal intensity in both ESI and APCI increased under zero voltage conditions when methanol was used as the makeup fluid and signal intensity was lower when elevated voltages were applied. The signal response increase was independent of the MS source configuration or design. Also, it was determined that methanol caused higher reference compound ionization due to its acidic functionality and acetonitrile suppressed ionization when used as a modifier. Thite concluded that the increased MS signal under zero voltage conditions may be caused by the outlet pressure or the acidity of CO_2 and that either APCI or ESI sources could be utilized. In SFC applications where

enhancement of MS signal is required, lower overall limits of detection (LOD) could be achieved with a reduction of the source high voltage.

Comparing SFC/MS & HPLC/MS Applications

Pinkston et al.[83] compared HPLC/MS to SFC/MS by screening a diverse pharmaceutical library and found SFC to be equivalent, reliable, and just as user-friendly as HPLC. Compound libraries are often solvated in dimethylsulfoxide (DMSO) to maintain compound stability; however, the authors observed positive and negative APCI ion suppression with sample solutions containing 10% DMSO and positive ion suppression at 5% DMSO. Chromatographic SFC peak shape was also improved for a broader range of polar and ionic analytes by adding ammonium acetate modifier. In this study, the number of target compounds that were successfully eluted and detected by SFC/MS and LC/MS were nearly equal. However, approximately 4% of compounds were detected by SFC/MS but not LC/MS, while 8% were detected by LC/MS but not SFC/MS. Pinkston found that compounds containing phosphorus functionalities such as phosphates, phosphonates, and bisphosphonates were the only functional groups undetected by SFC, which were observed by LC. In summary, the speed, environmental friendliness, cost efficiency, and orthogonality of SFC make it a suitable technique for high throughput screening of large and diverse drug-like compounds. Similar results were reported by Ventura,[68] Bolaños,[84] and Ripka,[85] who also found high throughput SFC useful for diverse and pharmaceutically relevant compounds using APCI mode.

Coupling semi-preparative SFC with electrospray mass directed fractionation enabled the collection of one pure fraction containing only the target compound.[69,86] Customized SFC/MS systems were created from a variety of commercial SFC and LC components, and displayed practical operational limitations. SFC purification flow rates were constrained to a maximum of 30 mL/min due to post decompression aerosol formation from the flow expansion and compatible fraction collection hardware. The hybrid instruments also required the use of multiple instrument control and data software systems. Novel SFC to MS splitters were required to direct small amounts of the SFC post-column flow to the mass spectrometer and correct for CO_2 – MeOH viscosity variations during the gradient, which causes the splitter to deliver a different split ratio to the MS. This had a noticeable effect on the signal response across the gradient, but reportedly did not affect the fraction recovery due to the negligible timing delay relative to the fraction peak width coming from the column at the flow rates used. Auxiliary solvents were optimized to obtain consistent MS response by using methanol-formic acid instead of water-methanol mixtures. Open bed fraction collection was also

complicated by the expansion of the mobile phase and, thus, required modifying the collectors or adding auxiliary fluids to maintain >85% recovery. The benefits of a SFC/MS purification approach results in higher purity fractionation, reduced fraction volumes, and fewer collected fractions.

Parallel SFC Applications

Maiefski et al. created a 4-channel, parallel mass directed SFC purification system that utilized a combination of commercial and custom built components. This system was capable of simultaneously sampling from 4 wells, detecting the target ion with a multi-channel source on a single TOF MS, and purifying each target in a plate to plate collection scheme. Mass directed purification allowed for the collection of target compounds in fewer fractions than a UV-directed purification approach. Their complex instrument also collected non-target products in a secondary "byproduct" plate to preserve the other well constituents for subsequent investigations. Although, the system was limited to collecting only enough volume that could fit in a 2 mL 96-well deep well plate, the throughput was well over 900 samples in 24 hours.[64,85] Parallel SFC/MS was also applied to chiral method development. Zeng et al. utilized custom software, 8 different chiral columns, and 6 different modifiers to select suitable chiral method conditions for a crude sample in less than 2 hours.[62,63] The supercritical fluid was split to the multiple columns using a 4 way manifold, multiplexed to a quadrupole mass spectrometer (MUX-4 MS) interface and pressure-controlled using a single backpressure regulator. Narrow bore ChiralPak® (AD-H & AS-H; ChiralCel® (OD-H & OJ-H), and Chirobiotic® (V, R, T, & Tag) columns were screened using ammonium acetate solutions of methanol, ethanol, or 2-propanol, and mixtures of ion pair reagents (ethyl- or methyl-sulfonic acids). The column layout is shown in Figure 2. This parallel analytical SFC/MS scheme could easily be adapted for other applications such as parallel achiral SFC/MS quantitation or purification.

The specificity of MS detection allowed Zhao et al.[87] to redefine the concept of parallel SFC. Using a single channel SFC/MS, increased throughput of chiral method development was achieved by simultaneously screening up to 12 enantiomeric pairs per injection, each pair having different molecular weights, through six different columns and four different modifiers (Figure 3). Each compound was monitored by extracted ion chromatograms until the separation reached acceptable chromatographic resolution. Custom software automated the mass spectral data processing, deconvoluted all the samples and printed separate reports. This strategy reportedly decreased chiral method screening time by 25%.

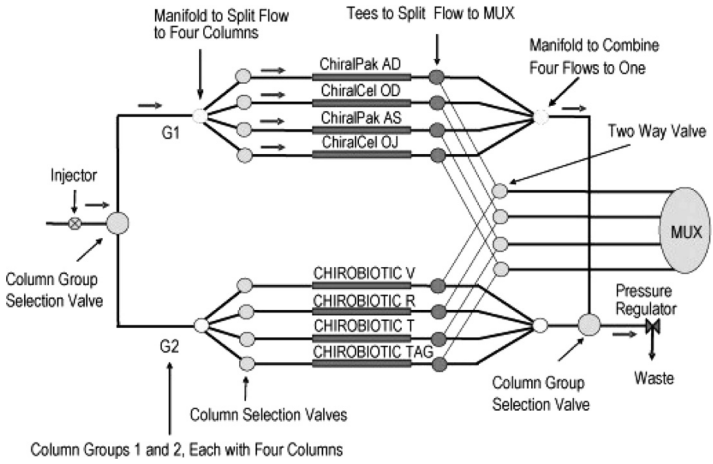

Figure 2. MUX-MS chiral column screening SFC/MS layout. (Reprinted from Ref. [62] with permission from Elsevier).

SFC Purification Applications

In 2004, Ventura et al. discussed their high throughput purification process, which utilized preparative scale SFC/UV, LC/UV, and LC/MS purification instruments coordinated by custom software sample tracking and data transfer. Purification techniques were selected with fast SFC/MS and LC/MS screening methods of the crude mixtures. The complimentary nature of this combination of chromatographic techniques enabled tens

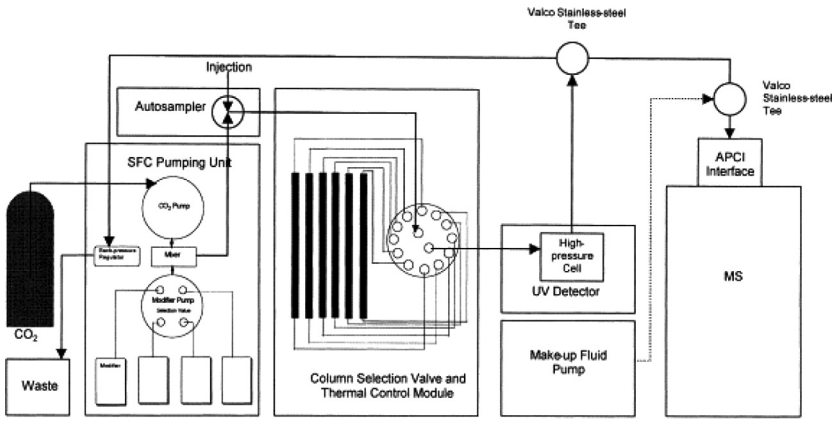

Figure 3. Schematic diagram of an SFC system. (Reprinted from Ref. [87] with permission from Elsevier).

of thousands of diverse compounds to be purified each year with success rates greater than 90%. It was demonstrated that the optimal purification process and efficient progression within the workflow depended on the successful integration of crude pre-analytical data and preparative instrumentation hardware to minimize attrition and maximize throughput.

White and Burnett utilized a 2-minute SFC/UV analysis method, which was scaled up to a 8.5-minute purification method using retention time calibrants and fraction collection time windows.[88] The analytical SFC used a $50 \times 4.6\,\text{mm}$ analytical column, which scaled to a $150 \times 21.2\,\text{mm}$ 2-ethylpyridine semi-preparative column, allowing for better separation of target compounds. The SFC conditions were validated with different compounds, including acids, bases, and neutrals, and showed strong analytical to preparative retention correlations ($R^2 = 0.9778$). The observed benefits of this SFC process demonstrated 77% faster analysis over HPLC, 18% faster SFC purification, and substantially reduced drying cycle times. Reconstructed chromatograms of the reference standard mixture comparing SFC and HPLC are shown in Figure 4.

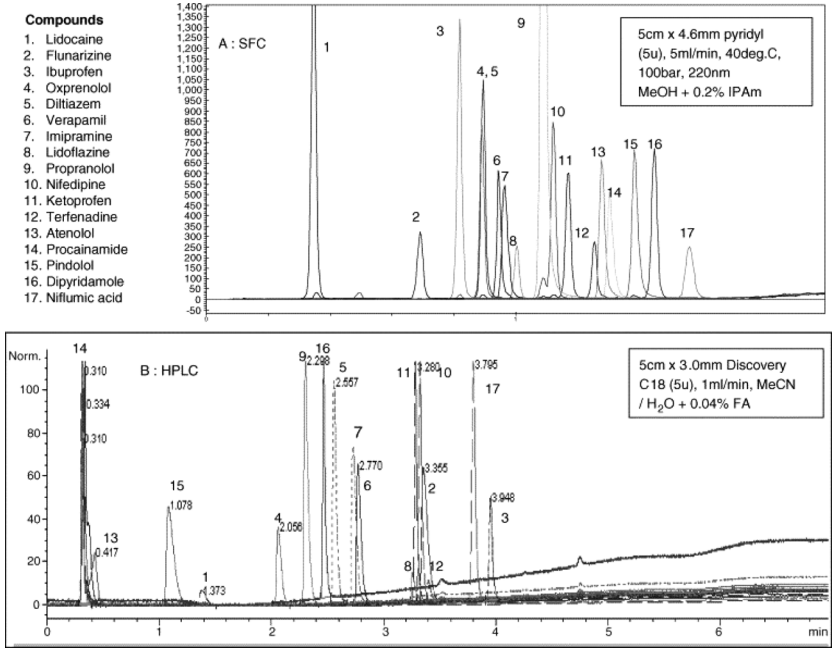

Figure 4. Comparison of drug standards by HPLC (6 min) versus SFC (2 min). (Reprinted from Ref. [88] with permission from Elsevier).

Bioanalytical SFC Applications

While SFC continues to gain acceptance in the traditional HPLC community, the applications have yielded some interesting and unique challenges. The clinical environment is an area with great potential for growth in SFC applications. Recent SFC bioanalytical applications have been reported for pharmacokinetic studies. Coe et al. reported the development of a high throughput SFC-MS/MS bioanalytical assay for R/S warfarin in human plasma.[89] Using tandem mass spectrometric techniques coupled to SFC, the authors were able to achieve higher MS sensitivities, significantly improved resolution of the enantiomer separation, and a cycle time that was twice as fast when compared to their previous HPLC-MS/MS method. In another report, Hoke et al. applied SFC technology to the ultra high throughput quantitation of dextromethorphan in human plasma. Using selected reaction monitoring and a unique injection scheme that sequentially loaded 8 injection ports, they achieved analysis times of 12 seconds and therefore could complete a 96 well plate every 10 minutes.[90]

A more recent SFC application was reported for two-dimensional (2D) chromatography, where peak capacity was greatly increased to separate complex samples. This coupled separation technique applies the effluent from one separation system to another, greatly enhancing the overall separation capacity. Francois et. al. developed a novel hyphenated SFC-RPLC system that performs orthogonally, beyond that afforded by comprehensive 2D HPLC for natural product samples.[91] This 2D chromatographic system was designed to mirror the concept of solid phase trapping during online coupling of SFE and HPLC. In this article, the authors used their 2D SFC-LC system to separate lemon oil extracts, whose numerous constituents are a mixture of psoralens and coumarins, making one-dimensional separation difficult. The sample eluted in the first dimension through the four SFC cyanopropyl silica columns connected in series using ethanol modified CO_2 as the SFC mobile phase. After the first dimension, chromatographic peaks were transferred to a RPLC column via a switching valve containing C18 ''trapping'' columns. During this transfer, supercritical CO_2 was decompressed and displaced by water, which forced the trapped sample to flow through the interface to the second dimension LC flow path. Next, the RPLC separation used a C18 column with an acetonitrile: water gradient. Since the 2D SFC-LC interface offered higher orthogonality compared to 2D HPLC, peak capacity was greatly increased, resulting in much higher separation peak capacity and resolution.

An innovative area for high throughput SFC is lipidomics, or lipid metabolomics. Lipids have many functions and are vital components of living organisms. They are required for maintenance of cell structure,

act as a form of energy storage, and provide ideal conditions for membrane protein interactions. However, abnormal levels of some lipids, such as cholesterol, are associated with a host of diseases: heart disease, obesity, diabetes, and atherosclerosis.[92] The separation, detection, and component analysis for a range of diverse and bioactively significant lipids is difficult due to the diverse molecular structures, variable polarities, and poor chromophores. Bamba et al.[93] developed a high speed analytical SFC/MS using ESI ionization, which enables the characterization of lipid components present in a complex mixture. They reported that a cyanopropyl column and methanol with 0.1% ammonium formate additive was successful for high throughput screening of lipids, while the octadecylsilated (ODS) column further enabled the separation and identification of the various lipid components. The significance to the field of lipidomics research is that SFC overcomes the difficulties of separating and, thus enabling, the identification of mixtures of complex lipids such as phospholipids, glycolipids, neutral lipids, and sphingolipids. There are other published reports using supercritical fluids for phospholipid analysis by Yip et al.,[94] but the analysis was performed using supercritical fluid extraction (SFE) rather than SFC.

The isolation and quantitation of estrogens and their related metabolites are important in breast cancer studies. The isolation and quantitation of these metabolites may help researchers determine their respective roles and mechanisms in cancer development and tumor initiation.[95] Due to a high degree of similarity in their structures, separation of these metabolites is difficult to accomplish. To exploit the high speed and high efficiency capabilities of SFC, Xu et al.[96] successfully developed a highly sensitive, high throughput SFC with tandem mass spectrometry for the separation and analysis of estrogen metabolites. These metabolites were derivatized with dansyl chloride to increase APCI sensitivity, and were injected onto dual columns (150×2.1 mm cyanopropyl and diol) connected in series. Separation of all 15 metabolites was achieved using a methanol gradient, and was found to be faster by SFC/MS than HPLC/MS by nearly a factor of 7. This accomplishment has potential impact in the clinical environment, which requires a high throughput chromatographic technique capable of simultaneous separation of a large number of estrogens and their metabolites in biological samples.

In the field of proteomics, the separation, identification, and characterization of complex mixtures of peptides is useful for biological drug target identification (peptide based drugs) and validation in physiological fluids. Peptide analysis requires high throughput chromatographic techniques and LC/MS has been the dominant technique in the proteomics field. However, Zheng et al. developed a SFC/MS method for the characterization of larger polypeptides containing a mixture of both acidic and basic residues.[97] They were able to successfully elute and separate

a group of peptides up to 40 residues in length using a non-endcapped ethylpyridine silica column. In addition, they determined that a TFA additive was required in the methanol modifier, since the acidity of TFA causes the polypeptides to elute and produce SFC/MS peak intensities sufficient for identification purposes. Zheng suggested that future 2D chromatographic analyses could alternatively use SFC in place of normal phase HPLC in the second dimension of peptide degradation applications where complex peptides usually result.

Protein-protein interactions are difficult to determine since the conformation of the protein may be altered upon reaction with hydroxyl radicals on its surface. These hydroxyl radicals serve to react with surface exposed residues, thus enabling the identification of the modification sites by mass spectrometry. Exchanging hydrogen for deuterium on the protein surface not only allows for identification of interaction sites, but no conformational changes result. This is typically done with the protein in a buffered solution containing D_2O, where the deuterium exchanges with solvent-accessible hydrogens. One drawback of this technique is the deuterated protein back exchanges during the separation and analysis of the protein, which negatively impacts the determination of the interaction sites. Increasing the speed of the HPLC analysis in order to reduce the protein hydrogen-deuterium (H/DP) back exchange did not eliminate this issue. As shown in Figure 5, Emmett et al. replaced the HPLC separation phase with SFC, since CO_2 does not contribute to

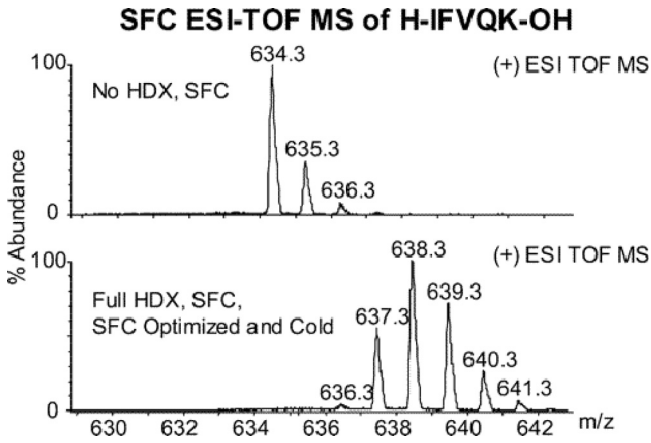

Figure 5. ESI-TOF mass spectra following SFC of the pentapeptide, IFVQK. (Top) 10-μL injection at 4 pM/μL undeuterated peptide to demonstrate that the peptide was retained on the SFC column. (Bottom) Fully deuterated sample (loaded at the same concentration) that had been stored in deuterated buffer for several days. (Reprinted from Ref. [98] with permission from ACS).

H/DP back exchange.[98] Preliminary studies using a rapid SFC/MS separation method indicated that protein solution phase H/DP exchange was further reduced and shows promise as a technique for more clearly determining protein-protein interactions.

CONCLUSION

High throughput SFC can be utilized in the same areas as HPLC and provides added benefits of high resolution, low solvent consumption, and fast analysis times. These advantages, combined with comprehensive workflow definition and design, will ensure that SFC maintains relevance into the foreseeable future. This technology can be applied broadly from pharmaceuticals ranging from antibiotics to neoplastic drugs. SFC has answered the HT demands of combinatorial chemistry and is beginning to show promising bioanalytical applications for the analysis of cell membrane, lipids, hydrophobic membrane proteins, and cholesterols. Emerging application areas include metabolomics, where SFC is showing promising results in the study of novel non-polar metabolites, biomarker identification, and regulation. Future applications will take advantage of SFC with its increased separation efficiency needed to study these complex mixtures.

REFERENCES

1. Lesellier, E. *Retention mechanisms in super/subcritical fluid chromatography on packed columns*; Journal of Chromatography Library: Elsevier, Amsterdam, 2008.
2. Berger, T.A. *Packed Column SFC*; Royal Society of Chemistry: London, 1995.
3. Taylor, L.T. Supercritical fluid chromatography for the 21st century. J. Supercrit. Fluids. **2009**, *47* (3), 566–573.
4. Berger, T.A. Analytical and semipreparative supercritical fluid chromatography in drug discovery. Drugs Pharm. Sci. **2004**, *138* (Supercritical Fluid Technology for Drug Product Development), 497–538.
5. Air Gas Product Data. http://www.airgas.com/content/products.aspx?id=9002002000000 (accessed January 20, 2009).
6. U.S._Department_of_Energy, Table H.1co2 World Carbon Dioxide Emissions from the Consumption and Flaring of Fossil Fuels, 1980–2006. http://www.eia.doe.gov/iea/carbon.html
7. United_States_Environmental_Protection_Agency, EPA420-F-05–001 - http://www.epa.gov/oms/climate/420f05001.htm. February 2005.
8. West, C.; Lesellier, E.A. Unified classification of stationary phases for packed column supercritical fluid chromatography. J. Chromatogr., A **2008**, *1191* (1–2), 21–39.

9. Okamoto, D.; Hirata, Y. Development of supercritical fluid extraction coupled to comprehensive two-dimensional supercritical fluid chromatography (SFE-SFCxSFC). Anal. Sci. **2006**, *22* (11), 1437–1440.
10. Staskiewicz, S.; Jones, A.; Melillo, D. Supercritical fluid chromatography and radiolabeled compound synthesis. J. Labeled Compd. Radiopharm. **2007**, *50* (5–6), 629–633.
11. Mukherjee, P.S. Validation of direct assay of an aqueous formulation of a drug compound AZY by chiral supercritical fluid chromatography (SFC). J. Pharm. Biomed. Anal. **2007**, *43* (2), 464–470.
12. Zhao, Y.; Pritts, W.A.; Zhang, S. Chiral separation of selected proline derivatives using a polysaccharide-type stationary phase by supercritical fluid chromatography and comparison with high-performance liquid chromatography. J. Chromatogr. A. **2008**, *1189* (1–2), 245–253.
13. Stringham, R.W. Chiral separation of amines in subcritical fluid chromatography using polysaccharide stationary phases and acidic additives. J. Chromatogr. A. **2005**, *1070* (1–2), 163–170.
14. Han, S.; Row, K. Chiral separation of ibuprofen by supercritical fluid chromatography. Chin. J. Chem. Eng. **2005**, *13* (6), 741–746.
15. del Nozal, M.J. Chiral separation of omeprazole and several related benzimidazoles using supercritical fluid chromatography. J. Sep. Sci. **2004**, *27* (12), 1023–1029.
16. Toribio, L. Chiral separation of some triazole pesticides by supercritical fluid chromatography. J. Chromatogr. A. **2004**, *1046* (1–2), 249–253.
17. Guebitz, G.; Schmid, M.G. Chiral separation principles in chromatographic and electromigration techniques. Mol. Biotechnol. **2006**, *32* (2), 159–179.
18. Matthijs, N.; Maftouh, M.; Vander Heyden, Y. Chiral separation strategy in polar organic solvent chromatography and performance comparison with normal-phase liquid and supercritical-fluid chromatography. J. Sep. Sci. **2006**, *29* (10), 1353–1362.
19. Gubitz, G.; Schmid, M.G. Chiral separations. Kirk-Othmer Sep. Technol. (2nd Ed.), **2008**, *1*, 553–579.
20. Ward, T.J. Chiral separations. Anal. Chem. **2006**, *78* (12), 3947–3956.
21. Ward, T.J.; Baker, B.A. Chiral Separations. Anal. Chem. **2008**, *80* (12), 4363–4372.
22. Mangelings, D.; Vander Heyden, Y. Chiral separations in sub- and supercritical fluid chromatography. J. Sep. Sci. **2008**, *31* (8), 1252–1273.
23. Phinney, K.W.; Stringham, R.W. Chiral separations using supercritical fluid chromatography, in *Chiral Separation Techniques,* Third Edition; VCH-Wiley: Caterbury, 2007, 137–154.
24. Beard, A.M. Chiral SFC and HPLC to support "high throughput" process research. Abstracts of Papers, 234th ACS National Meeting, Boston, MA, United States, August 19–23, 2007: p. ANYL-408.
25. Biba, M. Chiral SFC in pharmaceutical process research: From analytical to milligrams to kilograms. Abstracts of Papers, 230th ACS National Meeting, Washington, DC, United States, Aug. 28-Sept. 1, 2005: p. ANYL-279.

26. Biba, M. Chiral supercritical fluid chromatography (SFC) for analysis of Active Pharmaceutical Ingredients. Abstracts, 39th Middle Atlantic Regional Meeting of the American Chemical Society, Collegeville, PA, United States, May 16–18, 2007: p. MARM-233.
27. Hsieh, Y. Chiral supercritical fluid chromatography/tandem mass spectrometry for the simultaneous determination of pindolol and propranolol in metabolic stability samples. Rapid Commun. Mass Spectrom. **2005**, *19* (21), 3037–3041.
28. Andersson, S.; Nelander, H.; Oehlen, K. Preparative chiral chromatography and chiroptical characterization of enantiomers of omeprazole and related benzimidazoles. Chirality **2007**, *19* (9), 706–715.
29. Wu, D.-R. Preparative chiral separations of racemic mixtures using supercritical fluid chromatography. Abstracts of Papers, 226th ACS National Meeting, New York, NY, United States, September 7–11, 2003: p. ANYL-175.
30. Wu, D.-R. Preparative chiral separations of racemic mixtures using supercritical fluid chromatography. Abstracts of Papers, 230th ACS National Meeting, Washington, DC, United States, Aug. 28-Sept. 1, 2005: p. ANYL-282.
31. Welch, C.J. Preparative chiral SFC as a green technology for rapid access to enantiopurity in pharmaceutical process research. LCGC NA **2004**, *23* (1), 16,18, 22, 24, 26–29.
32. Welch, C.J. Preparative chiral SFC as a green technology for rapid access to enantiopurity in pharmaceutical process research. LC-GC Eur. **2005**, *18* (5), 264–266, 270, 272.
33. Stringham, R. Preparative chiral SFC separation of basic compounds using alkylsulfonic acid additives. Abstracts of Papers, 228th ACS National Meeting, Philadelphia, PA, United States, August 22–26, 2004: p. IEC-007.
34. Miller, L.; Potter, M. Preparative chromatographic resolution of racemates using HPLC and SFC in a pharmaceutical discovery environment. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. **2008**, *875* (1), 230–236.
35. Rajendran, A.; Mazzotti, M.; Morbidelli, M. Preparative chromatography at supercritical conditions. Adsorpt. Sci. Technol. Proc. Pac. Basin Conf., 3rd, Kyongju, Republic of Korea, May 25–29, **2003**, 204–208.
36. El-Saeid Mohamed, H. Pesticide residues in canned foods, fruits, and vegetables: The application of supercritical fluid extraction and chromatographic techniques in the analysis. Sci. World J. **2003**, *3*, 1314–26.
37. Mmualefe, L.C.; Torto, N.; Huntsman-Mapila, P.; Mbongwe, B. Supercritical fluid extraction of pesticides in sediment from the Okavango Delta, Botswana. Water SA. **2008**, *34* (3), 405–410.
38. Cole, J.; Dolak, L.A.; Lefler, J.L. The chiral resolution of an herbicidal product by SFC. Chim. Oggi. **2007**, *25* (Suppl.), 34–36.
39. Boulaid, M. Assessing supercritical fluid extraction for the analysis of fipronil, kresoxim-methyl, acrinathrin, and pyridaben residues in melon. J. Environ. Sci. Health, Part B. **2007**, *42* (7), 809–815.

40. Tribaldo, E.B. Chromatographic determination of carbamate pesticides in environmental samples, in *Chromatographic Analysis of the Environment,* Third Edition; Vol. 83, Chromatogr. Sci. Ser.; Marcel Dekker, Inc.: NY, 2006, 889–934.
41. Maione, M.; Mangani, F. Chromatographic analysis of insecticides chlorinated compounds in water and soil, in *Chromatographic Analysis of the Environment,* Third Edition; Vol. 83, Chromatogr. Sci. Ser.; Marcel Dekker, Inc.: NY, 2006, 803–840.
42. Rissato, S.R. Development of a supercritical fluid extraction method for simultaneous determination of organophosphorus, organohalogen, organonitrogen and pyretroids pesticides in fruit and vegetables and its comparison with a conventional method by GC-ECD and GC-MS. J. Braz. Chem. Soc. **2005**, *16* (5), 1038–1047.
43. Vagi, E. Phenolic and triterpenoid antioxidants from Origanum majorana L. herb and extracts obtained with different solvents. J. Agric Food Chem. **2005**, *53* (1), 17–21.
44. Rissato, S.R. Supercritical fluid extraction for pesticide multiresidue analysis in honey: Determination by gas chromatography with electron-capture and mass spectrometry detection. J. Chromatogr. A **2004**, *1048* (2), 153–159.
45. Venter, A.; Rohwer, E.R. Comprehensive two-dimensional supercritical fluid and gas chromatography with independent fast programmed heating of the gas chromatographic column. Anal. Chem. **2004**, *76* (13), 3699–3706.
46. Huehnerfuss, H.; Shah, M.R. Enantioselective chromatography-A powerful tool for the discrimination of biotic and abiotic transformation processes of chiral environmental pollutants. J. Chromatogr. A. **2009**, *1216* (3), 481–502.
47. Miller, L.; Potter, M. Preparative supercritical fluid chromatography (SFC) in drug discovery. Am. Pharm. Rev. **2008**, *11* (4), 112–117.
48. Leith, L. Preparative Supercritical Fluid Chromatography: From Method Development to Scale-up. Abstracts, 37th Northeast Regional Meeting of the American Chemical Society, Burlington, VT, United States, June 29-July 2, 2008: p. NERM-250.
49. Brunelli, C. Pharmaceutical analysis by supercritical fluid chromatography: optimization of the mobile phase composition on a 2-ethylpyridine column. J. Sep. Sci. **2008**, *31* (8), 1299–1306.
50. Abbott, E.; Veenstra, T.D.; Issaq, H.J. Clinical and pharmaceutical applications of packed-column supercritical fluid chromatography. J. Sep. Sci. **2008**, *31* (8), 1223–1230.
51. Alkio, M. Purification of pharmaceuticals and nutraceutical compounds by sub- and supercritical chromatography and extraction. VTT Pub. **2008**, *673*, 1–85.
52. Wu, D.-R.; Leith, L. The impact of chiral supercritical fluid chromatography in drug discovery. Am. Pharm. Rev. **2007**, *10* (6), 84–87.
53. Helmy, R. Improving sensitivity in chiral supercritical fluid chromatography for analysis of active pharmaceutical ingredients. Chirality. **2007**, *19* (10), 787–792.

54. Wang, Z. Development of supercritical fluid chromatography for chiral separations in pharmaceutical industry. Am. Pharm. Rev. **2007**, *10* (5), 96–100.
55. Zelesky, T.; Riley, F. Supercritical fluid chromatography benefits over HPLC for the purification of pharmaceutical chiral and achiral molecular targets. Abstracts of Papers, 234th ACS National Meeting, Boston, MA, United States, August 19–23, 2007: p. ANYL-372.
56. Ventura, M. High-throughput preparative process utilizing three complementary chromatographic purification technologies. J. Chromatogr. A. **2004**, *1036* (1), 7–13.
57. Zhao, Y. High-throughput drug discovery applications for supercritical fluid chromatography-mass spectrometry. Pharm. Disc. **2005**, *5* (2), 30, 32, 34, 36, 38–41.
58. Hochlowski, J. High-throughput purification: Triage and optimization. Chem. Anal. **2004**, *163*, 281–306.
59. Farrell, W.P. Implementation of supercritical fluid chromatography for high-throughput purification. Abstracts of Papers, 226th ACS National Meeting, New York, NY, United States, September 7–11, 2003: p. ANYL-174.
60. Hochlowski, J. Purification of HTOS libraries by supercritical fluid chromatography. J. Liq. Chromatogr. & Rel. Technol. **2003**, *26* (3), 333–354.
61. Ventura, M.C.; Farrell, W.P.; Aurigemma, C.M.; Greig, M.J. Packed column supercritical fluid chromatography/mass spectrometry for high-throughput analysis. Anal. Chem. **1999**, *71* (13), 2410–2416.
62. Zeng, L. Parallel supercritical fluid chromatography/mass spectrometry system for high-throughput enantioselective optimization and separation. J. Chromatogr. A. **2007**, *1169* (1–2), 193–204.
63. Laskar, D.B. Parallel SFC/MS-MUX screening to assess enantiomeric purity. Chirality **2008**, *20* (8), 885–895.
64. Maiefski, R.; Wendell, D.; Ripka, W.D.; Krakover, J.D. Apparatus and Method for Multiple Channel High Throughput Purification. U.P.T. Office, Editor; 2001; Ontogen Corporation.
65. West, C.; Lesellier, E. Characterisation of stationary phases in supercritical fluid chromatography with the solvation parameter model. V. Elaboration of a reduced set of test solutes for rapid evaluation. J. Chromatogr. A. **2007**, *1169* (1–2), 205–219.
66. Bui, H. Investigation of retention behavior of drug molecules in supercritical fluid chromatography using linear solvation energy relationships. J. Chromatogr. A. **2008**, *1206* (2), 186–195.
67. Dunkle, M.; Farrell, W.P.; Brunelli, C.; Van Hoek, E.; Sandra, P. Evaluation of the selectivity of novel stationary phases for supercritical fluid chromatography, In 2nd International Symposium on Green Chemistry. 2008: Zurich Switzerland.
68. Ventura, M.C.; Farrell, W.P.; Aurigemma, C.M.; Greig, M.J. Packed column supercritical fluid chromatography/mass spectrometry for high-throughput analysis. Part 2. Anal. Chem. **1999**, *71* (19), 4223–4231.
69. Wang, T.; Barber, M.; Hardt, I.; Kassel, D. Mass-directed fractionation and isolation of pharmaceutical compounds by packed-column supercritical fluid

chromatography/mass spectrometry. Rapid Commun. Mass Spectrom. **2001**, *15*, 2067–2075.
70. West, C.; Lesellier, E. Orthogonal screening system of columns for supercritical fluid chromatography. J. Chromatogr. A. **2008**, *1203* (1), 105–113.
71. Zheng, J.; Taylor, L.T.; Pinkston, J.D. Elution of cationic species with/without-without ion pair reagents from polar stationary phases via SFC. Chromatographia. **2006**, *63* (5–6), 267–276.
72. Brunelli, C. Development of a supercritical fluid chromatography high-resolution separation method suitable for pharmaceuticals using cyanopropyl silica. J. Chromatogr. A. **2008**, *1185* (2), 263–272.
73. West, C.; Fougere, L.; Lesellier, E. Combined supercritical fluid chromatographic tests to improve the classification of numerous stationary phases used in reversed-phase liquid chromatography. J. Chromatogr. A. **2008**, *1189* (1–2), 227–244.
74. West, C.; Lesellier, E. Separation of substituted aromatic isomers with porous graphitic carbon in subcritical fluid chromatography. J. Chromatogr. A. **2005**, *1099* (1–2), 175–184.
75. Lesellier, E. Overview of the retention in subcritical fluid chromatography with varied polarity stationary phases. J. Sep. Sci. **2008**, *31* (8), 1238–1251.
76. Gyllenhaal, O.; Edstroem, L.; Persson, B.-A. Ion-pair supercritical fluid chromatography of metoprolol and related amino alcohols on diol silica. J. Chromatogr. A. **2006**, *1134* (1–2), 305–310.
77. Zheng, J. *Supercritical fluid chromatography of ionic compounds*. Ph.D Thesis, Virginia Polytechic Institute, Blacksburg, Virginia; 2005.
78. Zheng, J. Effect of ionic additives on the elution of sulfonates and amine hydrochlorides in supercritical fluid chromatography. Abstracts of Papers, 230th ACS National Meeting, Washington, DC, United States, Aug. 28-Sept. 1, 2005: p. ANYL-277.
79. Bamba, T. High throughput and exhaustive analysis of diverse lipids by using supercritical fluid chromatography-mass spectrometry for metabolomics. J. Biosci. Bioeng. **2008**, *105* (5), 460–469.
80. Pinkston, J.D. Advantages and drawbacks of popular supercritical fluid chromatography/mass spectrometry interfacing approaches–a user's perspective. Eur. J. Mass Spectrom. **2005**, *11* (2), 189–197.
81. Sadoun, F.; Virelizier, H.; Arpino, P.J. Packed-column supercritical fluid chromatography coupled with electrospray ionization mass spectrometry. J. Chromatogr. A. **1993**, *647* (2), 351–359.
82. Thite, M.A. Ionisation in the absence of high voltage using supercritical fluid chromatography: A possible route to increased signal. Rapid Commun. Mass Spectrom. **2008**, *22* (22), 3673–3682.
83. Pinkston, J.D. Comparison of LC/MS and SFC/MS for screening of a large and diverse library of pharmaceutically relevant compounds. Anal. Chem. **2006**, *78* (21), 7467–7472.
84. Bolaños, B. SFC/MS in drug discovery at Pfizer, La Jolla. Intl. J. Mass Spectrom. **2004**, *238* (2), 85–97.
85. Ripka, W.C.; Barker, G.; Krakover, J. High-throughput purification of compound libraries. Drug Disc. Today **2001**, *6* (9), 471–477.

86. Zhang, X. Development of a mass-directed preparative supercritical fluid chromatography purification system. J. Comb. Chem. **2006**, *8* (5), 705–714.
87. Zhao, Y. Rapid method development for chiral separation in drug discovery using sample pooling and supercritical fluid chromatography-mass spectrometry. J. Chromatogr. A. **2003**, *1003* (1–2), 157–166.
88. White, C.; Burnett, J. Integration of supercritical fluid chromatography into drug discovery as a routine support tool. J. Chromatogr. A. **2005**, *1074* (1–2), 175–185.
89. Coe, R.A.; Rathe, J.O.; Lee, J.W. Supercritical fluid chromatography-tandem mass spectrometry for fast bioanalysis of R/S-warfarin in human plasma. J. Pharmaceut. Biomed. Anal. **2006**, *42* (5), 573–580.
90. Hoke, S.H.I.; Tomlinson, J.A., II.; Bolden, R.D.; Morand, K.L.; Pinkston, J.D.; Wehmeyer, K.R. Increasing bioanalytical throughput using pcSFC-MS/MS: 10 minutes per 96-well plate. Anal. Chem. **2001**, *73* (13), 3083–3088.
91. Francois, I. Construction of a new interface for comprehensive supercritical fluid chromatography * reversed phase liquid chromatography (SFC * RPLC). J. Sep. Sci. **2008**, *31* (19), 3473–3478.
92. Carrasco-Pancorbo, A.; Navas-Iglesias, N.; Cuadros-Rodríguez, L. From lipid analysis to lipidomics I. Modern lipid analysis. TrAC Trends Anal. Chem. In Press, Accepted Manuscript.
93. Bamba, T.; Atsuki, M.; Kazumasa, H.; Yoshihisa, N.; Akio, K.; Fukusaki, E. High throughput and exhaustive analysis of diverse lipids by using supercritical fluid chromatography-mass spectrometry for metabolomics. J. Biosci. Bioeng. **2008**, *105*, 460–469.
94. Yip Shiu-Hang, H.; Ashraf-Khorassani, M.; Taylor, L.T. Analytical scale supercritical fluid fractionation and identification of single polar lipids from deoiled soybean lecithin. J. Sep. Sci. **2008**, *31* (8), 1290–1298.
95. Clemens, M.G.P. Estrogen and the risk of breast cancer. N. Engl. J. Med. **2001**, *344*, 276–285.
96. Xu, X. Analysis of fifteen estrogen metabolites using packed column supercritical fluid chromatography-mass spectrometry. Anal. Chem. **2006**, *78* (5), 1553–1558.
97. Zheng, J. Feasibility of supercritical fluid chromatography/mass spectrometry of polypeptides with up to 40-Mers. Anal. Chem. **2006**, *78* (5), 1535–1545.
98. Emmett, M.R. Supercritical fluid chromatography reduction of hydrogen/deuterium back exchange in solution-phase hydrogen/deuterium exchange with mass spectrometric analysis. Anal. Chem. **2006**, *78* (19), 7058–60.

Invited Paper
Manuscript 6496F