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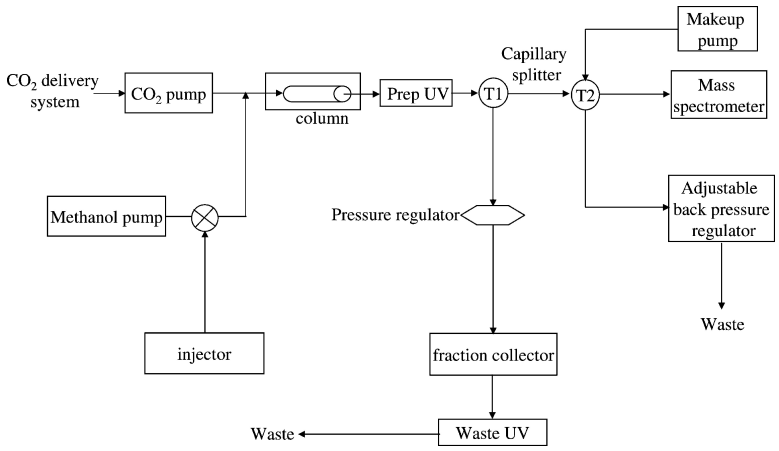


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# Development of a Mass-Directed Preparative Supercritical Fluid Chromatography Purification System

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In this paper, we report the development of a mass-directed supercritical fluid chromatography (SFC) purification system. We have addressed issues on software compatibility, the interface between the preparative SFC and the mass spectrometer, and fraction collection. Good peak shape and signal were achieved in the mass spectrometry (MS) trace, allowing accurate peak detection and reliable fraction collection. Simple modifications on a commercially available fraction collector enabled fractionation at atmospheric pressure with high recovery. The SFC/MS purification system has been used in support of high-throughput library purification and has been proven to be a valuable tool in complementing our reversed-phase high-performance liquid chromatograph (RP-HPLC/MS)-based technology platform.

## Introduction

In the past decade, successful development of automation for synthetic chemistry has gradually shifted the bottleneck of the drug discovery process to purification of library compounds.[1−3] The significance of high-throughput purification in support of parallel synthesis has been increasingly acknowledged, and continuous efforts have been made to enhance the technology and improve the overall purification efficiency.[4,5] Over the years, preparative reversed-phase high-performance liquid chromatography (RP-HPLC) has become the most widely used technique to purify crude reaction products from library synthesis because of its suitability for the compounds developed for pharmaceutical applications.[6,7] The purification process has evolved from initial UV-triggered or fixed-window collection to more widespread application of the mass-directed approach in which the selectivity of the mass spectrometer for target compounds allows for a controlled number of fractions.[2,8−12]

Along with the mainstream development on RP-HPLC-based purification, there has been an increasing interest in exploring the potential of using supercritical fluid chromatography (SFC) in the analysis and purification of compound libraries.[13−15] The supercritical fluid, typically consisting of compressed carbon dioxide ($CO_2$) and an organic modifier, such as methanol, has lower viscosity and higher diffusivity than liquid phases commonly used in LC. This enables higher flow rates without generating excessive backpressure. SFC is, in principle, a type of normal-phase chromatography in which the separation is based on polar interactions. It provides a selectivity that is different from RP-HPLC, thus allowing for an orthogonal approach in method development and 2-D separations for complex samples. To date, analytical SFC coupled with mass spectrometry (SFC/MS) has been widely implemented in the pharmaceutical industry for chiral and achiral compound analysis.[16] Over the past few years, SFC-based purification has also experienced a rapid growth in early drug discovery, especially for the separation of optical isomers.[17−19] SFC offers unique advantages in a purification process, such as reduced evaporation time, low solvent cost, and potential elimination of the use of mobile-phase additives.[20]

Currently, the application of SFC-based purification in drug discovery programs is primarily in the area of chiral separation.[16,21−23] Fractionation is typically controlled by a UV signal or a predetermined time window. Multiple fractions generated from each sample can be analyzed by a mass spectrometer using flow injection analysis. To minimize the number of fractions per sample and simplify the postpurification process, an indirect SFC/MS purification approach was introduced a few years ago.[20,24,25] In this approach, prepurification analysis is conducted on an analytical SFC/MS system. Retention times of the products are then downloaded to a preparative SFC system, where they are used to set time windows for fractionation, along with a UV threshold, if necessary. To ensure a linearity between the analytical and the preparative SFC systems, intersystem calibration is conducted daily by running a group of standard compounds on both systems and correlating their retention times. This approach addresses the deck capacity limitation associated with UV-based fractionation by controlling the number of fractions per sample and, hence, simplifies or completely eliminates the need for postpurification fraction tracking. However, it requires prepurification SFC analysis on all samples, and the reliability of the process is largely dependent on the linear correlation between the analytical and preparative SFC systems.

The advantages of mass-directed purification have been well-iterated, and its application has been widely imple-

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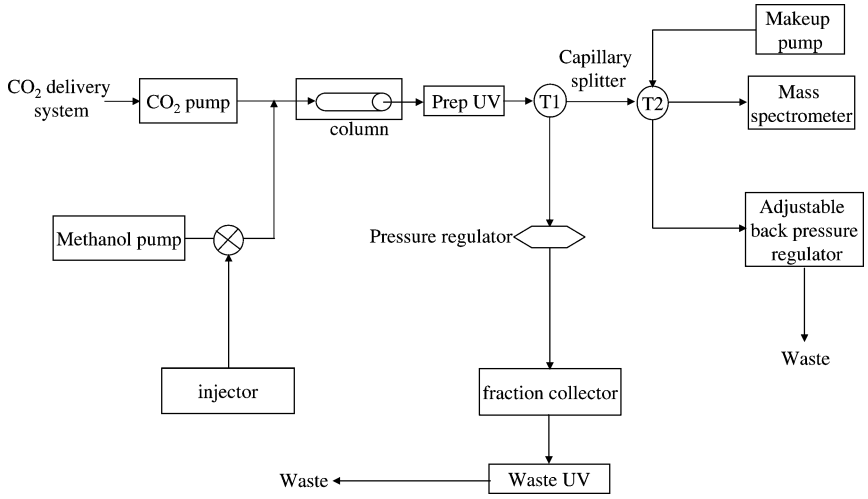


**Figure 1.** SFC/MS purification system diagram. Tubing internal diameters: auto sampler to column, column to prep UV, 0.01 in.; prep UV to T1, 0.02 in.; T2 to mass spectrometer: 0.0025 in.; T1 to pressure regulator, 0.03 in.; pressure regulator to fraction collector, 0.045 in.; fraction collector to waste UV, 0.08 in.

mented in HPLC-based purification.[8,26] However, the development of a mass-directed SFC purification system is not straightforward. Two of the biggest challenges are related to the SFC/MS interface and the fraction collector. The challenge in constructing an SFC/MS interface comes from a much greater pressure drop at the flow splitter between the SFC and the mass spectrometer, along with potential signal-to-noise ratio deterioration caused by phase separation.[27] The other challenge is the design of the fraction collector. The SFC mobile phase undergoes a drastic pressure drop after the pressure regulator. An aerosol formation in which the volume of the flow expands up to 500 times occurs during this process. This makes physical collection of the fraction much more challenging than in HPLC.

In 2000, Ontogen developed a mass-directed SFC purification system that allows up to four samples to be purified at one time.[28] Purified samples can be deposited back into a microtiter plate by using a disposable "expansion chamber" on a customized fraction collector. The reported flow rate was 12 mL/min with a cycle time of 5.5 min. In 2001, Zhu et al. developed a SFC/MS purification system using a modified Gilson fraction collector.[29] A piece of long, restrictive tubing was used to connect the SFC and the mass spectrometer to counter the large pressure drop. Fraction collection was conducted at atmospheric pressure with a piece of foil cover wrapped around the top of the collection tubes. The typical sample load was 5−15 mg on a 10 × 150 mm column with a flow rate of 15 mL/min. Overall recovery was 77% with the foil cover and 45−50% without.

These pioneer works have encouraged additional interest in the development of SFC/MS-based purification. However, to date, mass-directed SFC purification systems have not yet become commercially available. The main hurdles are software incompatibility and the hardware issues mentioned above.

We have developed a preparative SFC system that uses the MS signal as the fraction trigger and enables a "one-to-one" sample-to-fraction ratio, similar to our RP-HPLC/MS-based purification approach.[8] Through modifications on the fraction collector, we were able to collect fractions under atmospheric pressure with high recovery at flow rates up to 30 mL/min.

This preparative SFC/MS system has been used in library purification as a technology that is complementary to our RP-HPLC/MS-based purification platform. In this paper, we will report some of our successes in overcoming the issues mentioned above and demonstrate some preliminary results.

## Experimental

**Instrumentation.** The SFC/MS purification system consists of a Berger Manual Preparative SFC system (Mettler-Toledo AutoChem, Newark, DE), a Gilson 215 liquid handler with an 819 injection module (Madison, WI), a Waters 2757 fraction collector, and a Waters ZQ single quadrupole mass spectrometer (Milford, MA). The Berger Manual Preparative SFC system includes an electronic module, a separator module, two Varian pumps that deliver liquid $CO_2$ and the organic modifier, a Knauer variable wavelength UV detector operating at 254 nm (subsequently referred to as prep UV), a chiller, and a waste containment system. Liquid $CO_2$ is provided to the pump by a Berger SFC G700 $CO_2$ Gas Delivery System (Mettler-Toledo AutoChem). Four dewars, each with a capacity of 160 liters of liquid $CO_2$, are connected through an automatic switchover. A system schematic diagram is shown in Figure 1. A Shimadzu SP-10ADVP UV detector (Columbia, MD), equipped with an analytical flow cell and operated at 300 nm was used to analyze the MS split ratio.

**Materials.** Medical grade $CO_2$ was purchased from Airgas (Salem, NH). HPLC grade methanol was purchased from EMD Chemicals (Gibbstown, NJ). Brucine, carbamazepine, chlorpropamide, erythromycin, flavone, formic acid, ibuprofen, and noscapine were purchased from Sigma-Aldrich (St. Louis, IL). Library compounds were synthesized in-house and submitted to purification.

**SFC and MS Conditions.** Preparative SFC separations were conducted using 10 × 100 mm, 10-$\mu$m Princeton SFC Pyridine columns or 21.2 × 150 mm, 6 $\mu$m Berger Cyano columns. Flow rate was 5−30 mL/min. SFC column temperature was kept at 40 °C. Nozzle temperature was 60 °C,

and nozzle pressure was 100 bar. A 15-cm capillary splitter with a diameter of 25 $\mu$m was placed after the prep UV detector to divert a small portion of the flow to the mass spectrometer, which was then diluted by a 2 mL/min flow of makeup solvent before entering the mass spectrometer. The makeup flow consisted of methanol and 0.3% formic acid. The pressure at the end of the capillary splitter was controlled by an adjustable backpressure regulator (Alltech Associates, Inc., Deerfield, IL). A SPD-10AVP UV detector (subsequently referred to as waste UV), operating at 254 nm, was located after the fraction collector to monitor the collection.[8] The ZQ mass spectrometer was operated in electrospray ionization mode. The mass spectrometer scans from 190−800 amu with a 0.5-s scan time and a 0.1-s interscan delay.

**Sample Injection and Fraction Collection.** All commercially available compound samples were injected from methanol. Library compounds were injected from a mixture of methanol and dimethyl sulfoxide (DMSO). Injection volumes ranged from 50 to 1500 $\mu$L. The flow of methanol pushes the sample plug out of the injection loop and combines with the flow of $CO_2$ through a tee. In comparison with a setup in which the combined flow of $CO_2$ and methanol displaces the sample from the injector, this flow path works similarly to the "at-column dilution" setup, which increases the sample's volume loadability in gradient runs.[30] A Waters 2757 fraction collector was equipped with a two-way switching valve that was rated to 100 psi. Fraction detection peak type was set to "preparative". Purified fractions were collected into culture tubes that hold up to 16 mL of solution. These fraction collection tubes were arranged in custom-made 24-well collection racks.

**Weighing and Evaporation.** The fraction collection tubes were tared before purification. Solvent evaporation was conducted with Mega 980 Evaporators from Genevac Inc. (Valley Cottage, NY). After solvent evaporation, collection tubes with fractions were reweighed. Weighing was performed using a digital balance with 0.1-mg accuracy (SAG 285, $d$ = 0.01 mg, Mettler Toledo, Columbus, OH), controlled by a Bohdan automated weigher (Mundelein, IL).[8]

**Software Control.** The preparative SFC/MS system is controlled by two different software applications on two separate computers. Berger ProNTo software controls the SFC conditions, including gradient condition and run time, oven temperature, nozzle temperature and pressure. Sample injection, fraction collection, and MS data acquisition are controlled by Waters MassLynx 4.0 and FractionLynx 4.0. The communication between the two software applications was achieved by a contact closure that connects the SFC electronic module and the Gilson 215 auto sampler (Figure 2). A gradient run starts when the injection valve rotates on the Gilson 819 module. In setting up a sequence run with multiple samples, two identical sample lists need to be started simultaneously in both ProNTo and MassLynx.

## Results and Discussion

Several features of SFC-based purification make this technique attractive for the purification of early discovery compounds. The separation mechanism of SFC is radically different from RP-HPLC, allowing for a different selectivity that can be very valuable. SFC also addresses certain limitations associated with RP-HPLC-based purification, such as the relatively long turnaround time for evaporation and the significant cost of solvent and waste handling. To incorporate SFC-based purification into our high-throughput technology platform, we felt it important to have the capability of mass-directed fractionation, enabling a "one-to-one" sample-to-fraction ratio, which has made our RP-HPLC/MS purification strategy so successful. In this section, we will discuss some of the major issues we have addressed and the solutions we came up with to develop the preparative SFC/MS system.

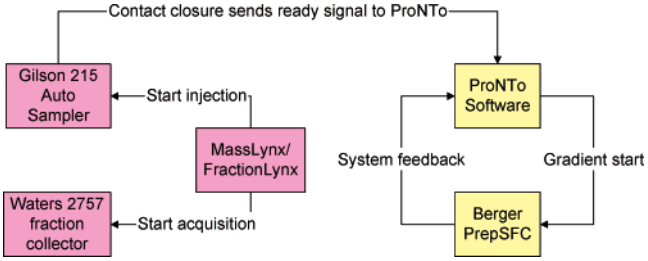


**Figure 2.** Software configuration.

**SFC/MS Interface Construction and MS Signal Optimization.** The SFC mobile phase undergoes a phase change at the pressure regulator. Before the pressure regulator, $CO_2$ and methanol coexist as super- or subcritical fluid under controlled pressure and temperature conditions. After the pressure regulator, a drastic pressure drop from 100 bar to near atmosphere triggers a phase separation in which $CO_2$ is converted to its gaseous state and separates from the liquid methanol.

The SFC/MS splitter design is demonstrated in Figure 1. The MS splitter is located before the pressure regulator where the pressure is maintained at 100 bar. At the temperature and pressure conditions at which a SFC system is normally operated, the density of the $CO_2$ and methanol mixture does not change significantly with different gradient conditions.[31] Therefore, the delay time, which is the time difference between peak detection at the mass spectrometer and fraction collection, remains largely unchanged in a gradient run as in the case of preparative HPLC/MS. As mentioned earlier, the large pressure drop across the capillary splitter is one of the most important factors to consider in designing the SFC/MS interface.[32] In an HPLC system, pressure drop occurs mostly across the column. The pressure at the MS splitter mainly comes from the tubing between the splitter and the fraction collector. However, in an SFC system, the pressure at the beginning of the capillary (SFC side) is close to the pressure at the pressure regulator, for example, 100 bar. To achieve a reasonable split ratio, an appropriate pressure needs to be maintained at the end of the capillary (mass spectrometer side) to counter the large pressure difference and to control the amount of sample going into the mass spectrometer, especially since the viscosity of the fluid is low.

To optimize the MS peak shape and signal-to-noise ratio, it is important to control the flow rate in the capillary splitter, which is primarily determined by the pressure drop across the capillary splitter, assuming the capillary's diameter,

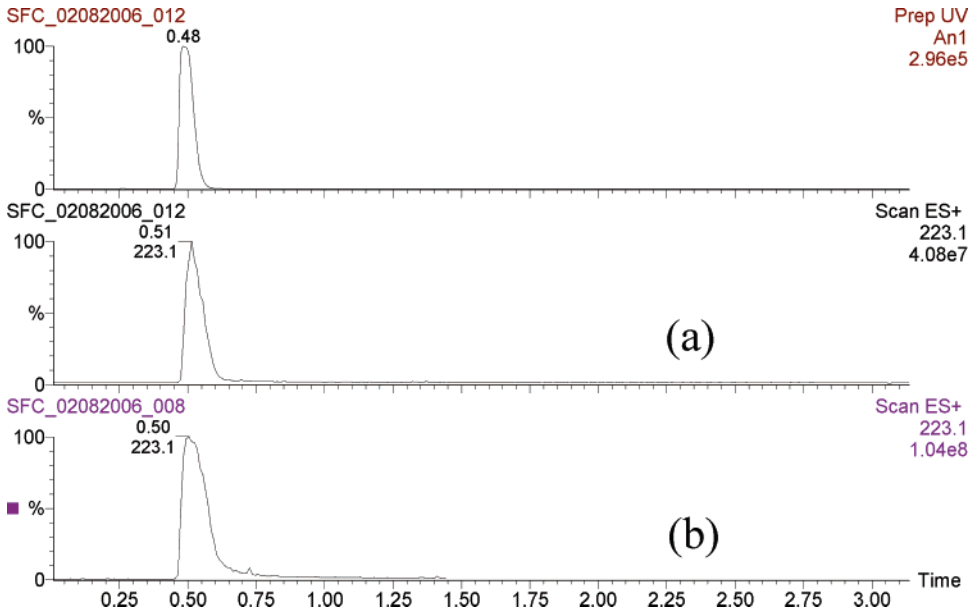


**Figure 3.** Chromatograms of flavone. Traces from top to bottom: prep UV trace, selected ion chromatograms with pressure drop across the capillary maintained at (a) 150, (b) 800 psi.

length and the viscosity of the liquid are constant. When the pressure drop is too low, a very limited amount of sample passes through the capillary splitter, causing low signal intensity in the MS trace. When the pressure drop is too high, too much material goes into the mass spectrometer, saturating the ion source and causing a broad peak shape in the MS, which can compromise fraction purity. Figure 3 shows two examples in which pressure drops across the capillary were 150 psi (Figure 3a) and 800 psi (Figure 3b). At a pressure drop of 150 psi, only 30% of band-broadening was observed between peaks in the prep UV and the MS traces. At a pressure drop of 800 psi, 50% of band-broadening was observed from the prep UV to the MS. Also noticed was a much higher MS signal intensity, indicating a possible saturation in the detector. In our study, well-focused peaks with reasonably good signal-to-noise ratios were achieved when the pressure drops across the capillary splitter were controlled at 100−600 psi.

In addition to the pressure drop, the viscosity of the eluent also affects the split ratio. This happens in a gradient run when the $CO_2$/methanol composition changes. To quantitatively analyze the split ratio at different pressure drops and $CO_2$/methanol compositions, we replaced the mass spectrometer with an analytical UV detector (subsequently referred to as analytical UV) and eliminated the split to the waste downstream from the capillary splitter. Pressure at the end of the capillary was controlled by an adjustable pressure regulator. The peak area in the analytical UV detector reflects the amount of the sample passing through the capillary. Results shown in Figure 4 indicate that the split ratio decreases during the gradient as the percentage of methanol and the viscosity of the mobile phase increase. It is important to note that this change in split ratio does not affect the delay time and, therefore, does not compromise the fraction recovery. This is because the change in travel time of the compound through the capillary splitter is negligible, as compared to the standard peak width of a fraction coming

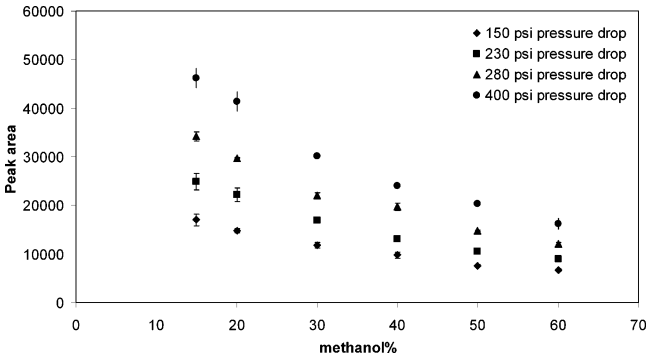


**Figure 4.** Flavone intensity at different $CO_2$/methanol compositions.

from the column. Figure 4 also demonstrates that the split ratio decreases with the overall pressure drop across the splitter, which is apparent in Figure 3 and was expected.

In electrospray positive mode, signal intensity in the MS trace increases with a higher percentage of $CO_2$ in the mobile phase. This trend reverses in the electrospray negative mode. We believe that there are two factors impacting the MS signal intensity in an SFC gradient run. One is the change of split ratio with the $CO_2$/methanol composition. The other is the pH change caused by the $CO_2$ in the flow going to the mass spectrometer.[33,34] A higher percentage of $CO_2$ decreases the flow's viscosity while increasing its acidity. In electrospray positive mode, this provides overall enhanced ionization efficiency. On the other hand, in electrospray negative mode, although the split ratio increases at higher $CO_2$ percentage, the increased acidity suppresses the ionization efficiency, causing overall decreased signal intensity. In a mass-directed purification application, it is important to have a reasonably consistent MS signal to ensure reliable collection. We have found that a makeup flow that contains a relatively high acid concentration but no water can minimize the impact on the MS signal intensity from $CO_2$ in the electrospray positive mode, which works for the vast majority of pharmaceutical

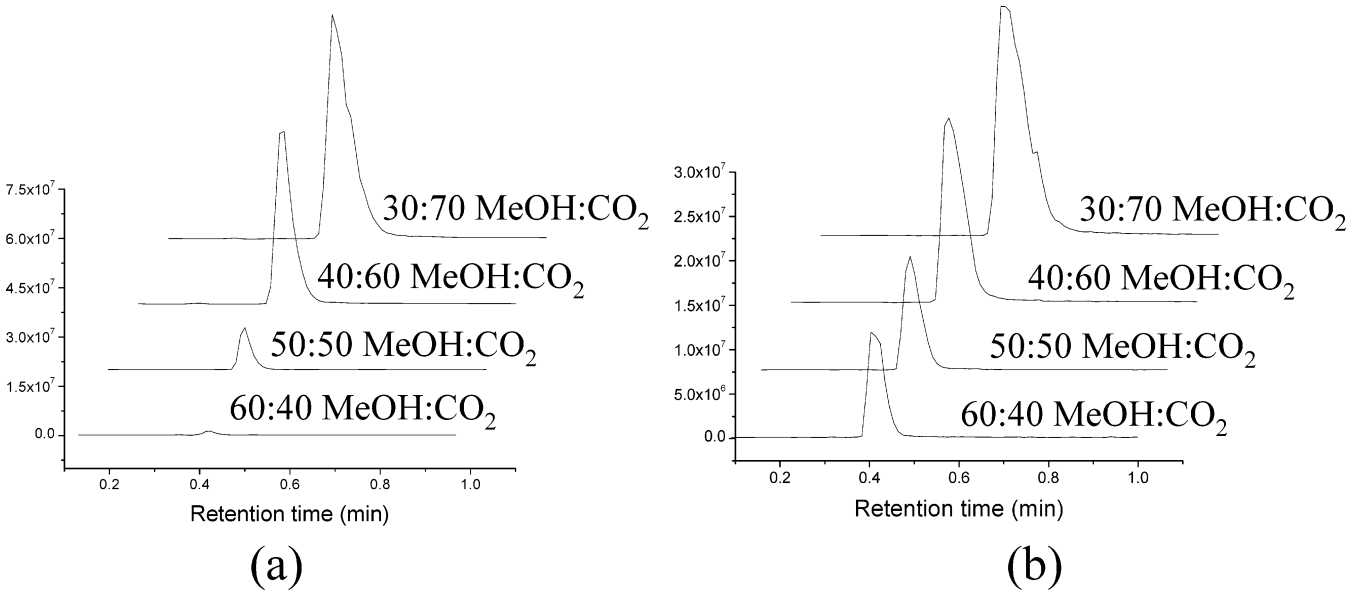


**Figure 5.** Selected ion chromatograms of flavone at different SFC mobile phase compositions. Makeup flow consists of (a) 90:10:0.3% methanol/water/formic acid and (b) 100:0.3% methanol/formic acid.

compounds. Figure 5 shows selected ion chromatograms of flavone at different $CO_2$/methanol compositions. When 90:10:0.3% methanol/water/formic acid was used as the makeup solvent, the MS signal intensity decreases rapidly with increased $CO_2$ percentage (Figure 5a). However, when 100:0.3% methanol/formic acid was used as the makeup solvent, the loss of MS signal intensity at higher methanol percentage was minimized (Figure 5b).

**Column Efficiency Optimization.** Due to the limited pressure rating on the collection valve (100 psi), the maximum flow rate used on the preparative SFC system was 30 mL/min, which is lower than the flow rates at which this type of instrument is normally operated. Under such circumstances, column efficiency has to be monitored, because extra column volume can have a significant impact on efficiency. To maximize column efficiency, we made flow path modifications to minimize the extra column volume. The tubing diameter between the auto sampler and the column and that between the column and the prep UV was changed from 0.03 to 0.01 in. (250 $\mu$m). A check valve, originally located between the injector and the column, was placed between the methanol pump and the auto sampler. The tubing diameter between the prep UV and the capillary splitter was 0.02 in. Figure 6 shows chromatograms acquired before and after these modifications and highlights the importance in optimizing the flow path to achieve optimized system efficiency.

**Waste UV Detector and Delay Time Assessment.** As mentioned in a previous paper on preparative RP-HPLC/MS, we routinely use a second UV detector in the waste line, downstream from the fraction collector.[8] This is used to assess the delay time and allows us to monitor the effectiveness of fractionation in real time. To be able to use a waste UV detector in an SFC/MS system, as well, would be highly desirable; however there are some major differences that need to be taken into account. At the back-pressure regulator, the SFC mobile phase undergoes a controlled decompression, generating a heterogeneous phase downstream. Because the proper operation of the SFC pressure regulator does not allow for significant downstream backpressure, a piece of large-bore tubing with a diameter of 0.08 in. was used between the fraction collector and the waste UV to ensure that the pressure did not exceed ~50−100 psi. In a RP-HPLC system, the usage of such a wide-bore tubing would have caused severe band-broadening issues; however in SFC, as is shown in Figure 7, minimal band-broadening was observed between the prep UV and the waste UV traces. We believe that this is mainly due to the drastic volume expansion of $CO_2$ upon decompression. When the peak volume becomes much larger, the extra column volume that had such a detrimental effect under liquid-phase conditions becomes negligible in SFC. Due to this phase separation, we also observed a reduced signal-to-noise ratio for the waste UV detector, as compared to that of the Prep UV. This is not unexpected, because the liquid phase is diluted by a large amount of gas. An elevated baseline is observed in the waste UV trace as the percentage of methanol increases. This could be related to refractive index issues in

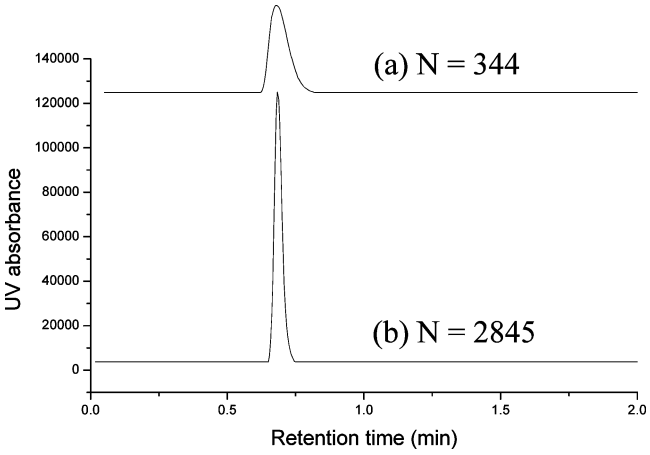


**Figure 6.** Column efficiency (a) before and (b) after modification made on system flow path. Plate count was calculated through retention times and peak widths at half height in isocratic condition.

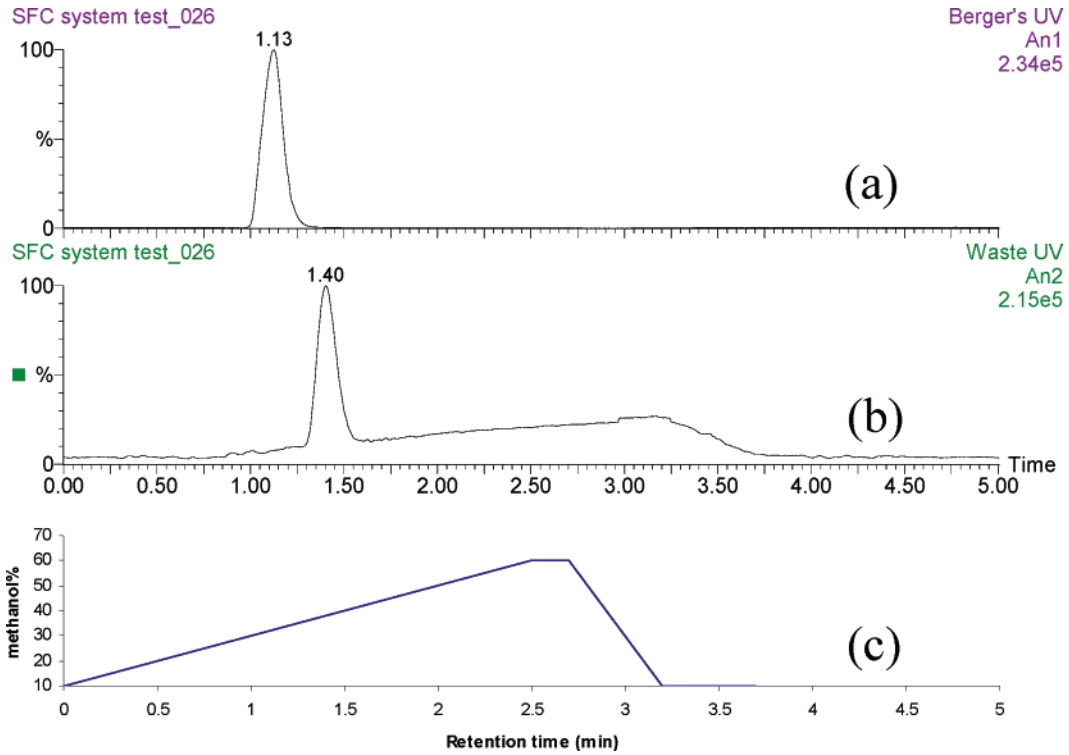


**Figure 7.** A gradient run: (a) prep UV trace, (b) waste UV trace, and (c) gradient condition.

the flow cell while dealing with a binary phase system. Despite the higher baseline noise in the waste UV trace, for most compounds, we found that it is feasible to use the waste UV in the SFC system to assess delay time and to monitor fraction collection in real time.

As mentioned earlier, a consistent delay time is critical in mass-directed fractionation. SFC mobile phase undergoes a massive volume expansion at the pressure regulator, resulting in different volume flow rates before and after. Consequently, the volume flow rate after the pressure regulator varies in a gradient run as $CO_2$/methanol composition changes. To minimize the impact this might have on the delay time, the tubing volume between the pressure regulator and the fraction collector should be kept relatively small. The delay time on our SFC system was determined to be 4.5 s by comparing the MS and the waste UV traces. Collections conducted at different gradient conditions indicate that the delay time remains reasonably constant in a typical gradient of 10−60% of methanol in $CO_2$. Figure 8 shows examples in a gradient run when correct and incorrect delay times were used.

**Fraction Collection.** Currently, commercially available fraction collectors for preparative SFC systems consist of pressurized collection chambers. In one automated system, a Bohdan robot moves collection tubes into the chamber prior to collection and replaces them back to collection racks after the collection.[20,35] The pressurized collection chamber minimizes aerosol formation and ensures high collection recovery. However, the Bohdan robots are expensive and incompatible with MassLynx and, therefore, cannot be easily incorporated into our mass-directed purification platform.

Ideally, we would like to retain our overall process and simply replace the RP-HPLC separation with SFC separation. On the basis of this idea, we successfully modified a Waters 2757 fraction collector to enable SFC fraction collection at atmospheric pressure. We believe that the major reason for aerosol formation, which causes sample losses, is the flow's high linear velocity at the collection needle tip. For a normal fraction collector, the steel collection needle acts almost as a nebulizer when the small amount of methanol is expelled by the large volume of $CO_2$. We replaced the original collection needle with a 3.2-mm-diameter Teflon tubing, significantly reducing the linear velocity of the gas/liquid mixture. The fractions are dispensed ∼1.5 cm below the rim of the collection tube opening so that the small droplets formed at the tip of the tube do not splash outside the collection tube. The original rinse station was enlarged and vertically extended to prevent splashing during collection needle wash. High recoveries at flow rates up to 30 mL/min were achieved, as shown in Figure 9, indicating that the eluent was very effectively collected and that losses to aerosol formation were very low, if existent at all. In addition, no measurable cross-contamination was found between neighboring tubes. Recoveries of a series of commercially available compounds are shown in Figure 10.

**Preliminary Investigation on Column Loadability.** In RP-HPLC, the loading capacity for acidic or basic compounds can be adjusted by changing the pH in the mobile phase. This is different in SFC because we deal with a nonaqueous medium. Because of a range of suitable stationery phases available, SFC separation can be conducted without any mobile phase additive. Historically, SFC had suffered from poor peak shape and low loadability, as compared to RP-HPLC, particularly in the cases of basic compounds.[36] A breakthrough on chromatographic performance for basic compounds was brought by the introduction of a 2-ethylpyridine phase. When compared to traditional bonded polar phases such as the cyano or diol materials, the

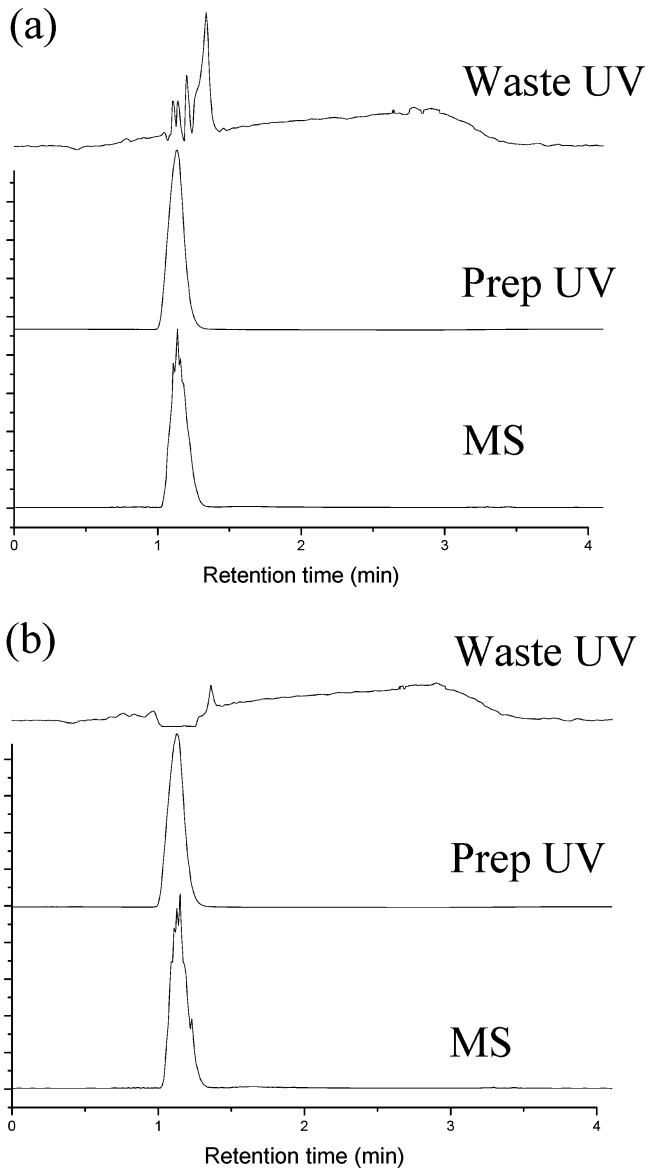


**Figure 8.** Fraction collection at different delay times. (a) Collection performed with an incorrect delay (8 s). (b) Collection performed with a correct delay (4.5 s).

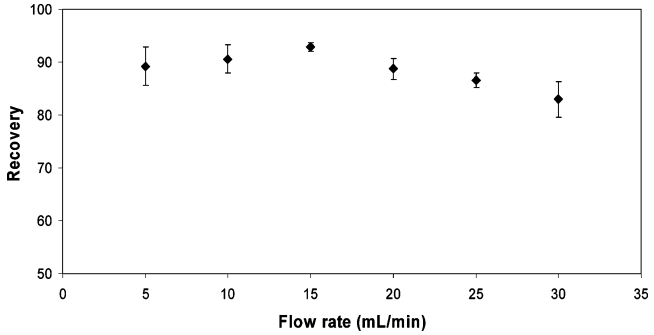


**Figure 9.** Recoveries over a range of flow rates.

2-ethylpyridine phase offers greatly improved peak shape and overall column efficiency for basic compounds without any amine additives.[36,37] Figure 11 shows injections of noscapine on RP-HPLC and SFC with increasing mass loadings. Overall, peak shape and asymmetry factors from SFC were better than the ones from the RP-HPLC. On the

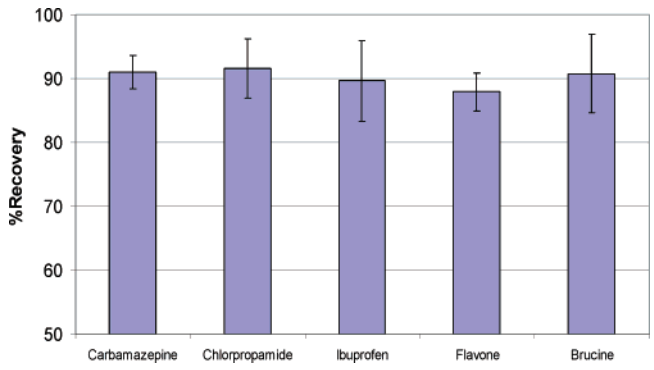


**Figure 10.** Recoveries of a series of druglike compounds. Carbamazepine, chlorpropamide, flavone, and brucine were purified on a 2-ethylpyridine column (10 × 100 mm, 10 $\mu$m). Ibuprofen was purified on a cyano column (21.2 × 150 mm, 6 $\mu$m).

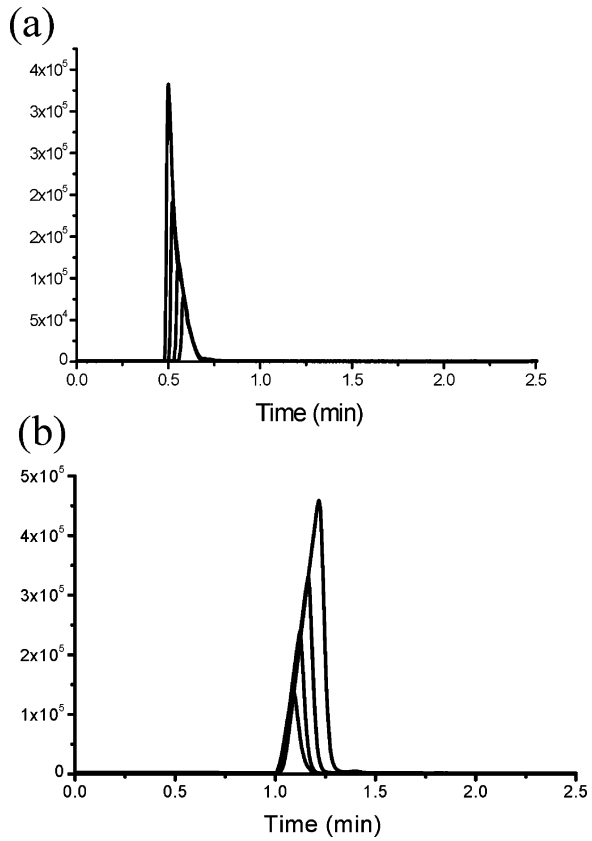


**Figure 11.** Injections of noscapine at different mass loadings. Traces from top to bottom: 40, 20, 10, and 5 mg. Injection volume: 0.5 mL from methanol. (a) RP-HPLC. 5−95% acetonitrile in $H_2O$ in 1.75 min with a cycle time of 2.5 min. Mobile phase contains 0.1% TFA. Column: 20 × 50 mm Maccel C18. Flow rate: 88 mL/min. (b) SFC. 10−60% methanol in $CO_2$ in 2.5 min with a cycle time of 4 min. Column: 10 × 100 mm 2-ethylpyridine. Flow rate: 20 mL/min.

HPLC system (Figure 11a), the mass overloading effect was observed at a relatively low quantity (5 mg), indicated by reduced peak capacity and the formation of a right-triangular peak with decreased retention time. Peak capacity from SFC, shown in Figure 11b, was fairly consistent up to a larger quantity (20 mg). In addition, the collection volume in SFC is much smaller than that of HPLC, since a major component of the mobile phase is $CO_2$ , which evaporates immediately upon collection, enabling a much wider maximum collection window compared to that of HPLC.

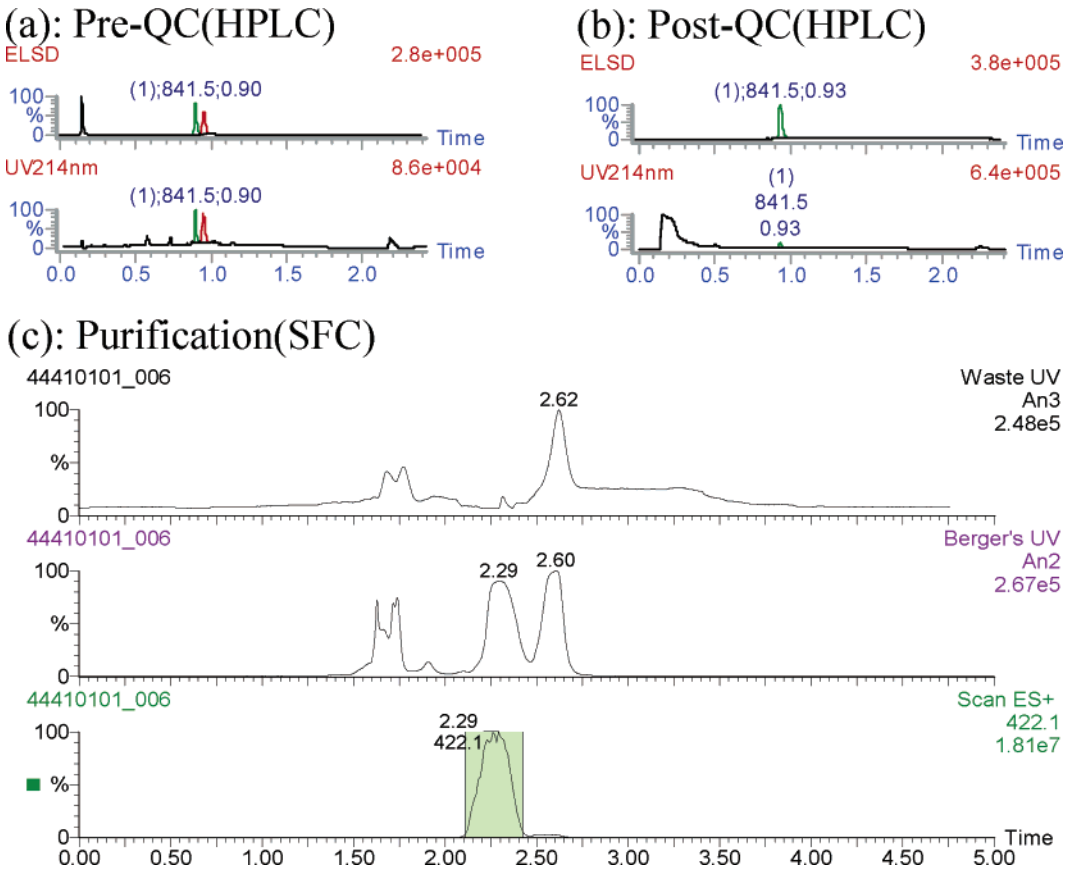


**Figure 12.** Library compound purification: (a) pre-QC and (b) post-QC conducted on RP-HPLC/MS and (c) purification conducted on SFC/MS.

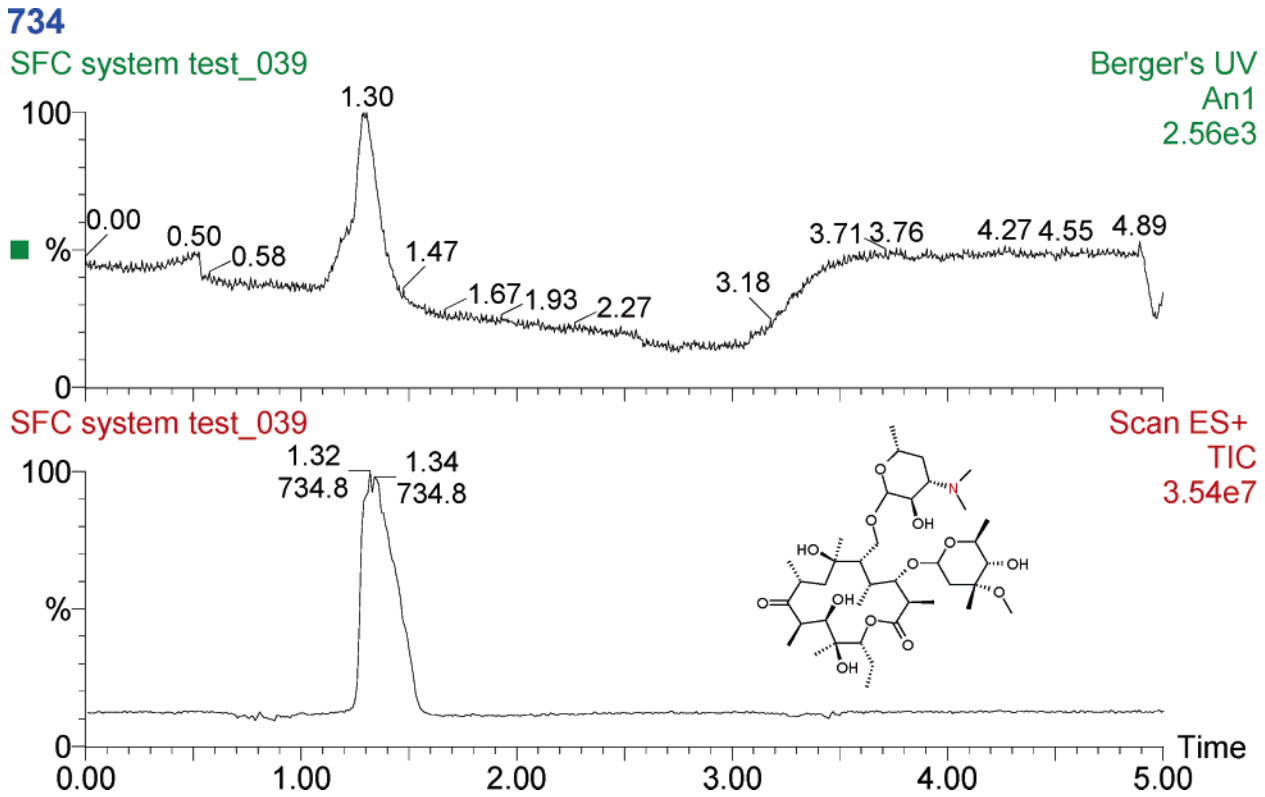


**Figure 13.** Chromatogram of erythromycine. Injection volume: 1 mL in methanol. Mass loading: 20 mg. Gradient: 10−60% methanol in $CO_2$ in 2.5 min. Column: 10 × 100-mm 2-ethylpyridine column.

**Application of Mass-Directed SFC Purification.** High-throughput library purification platforms today frequently rely on RP-HPLC/MS technology.[8] RP-HPLC is well-suited to handle the quantities and polarities of compounds in drug

discovery programs, whereas mass-directed purification can be used to control the number of fractions per sample and, hence, simplifies the overall process.[26] Implementations of short columns, high flow rates, and rapid method optimization have enabled us to provide fast and reliable support in library purification.[8,38] The incorporation of a preparative SFC/MS system in our purification platform has been driven by the need to enhance the diversity of purification technology and improve the overall process. Preparative SFC is particularly valuable in supporting lead optimization programs, in which libraries with extensive diversity are produced with a need for rapid turnaround times. In RP-HPLC-based purification, overnight evaporation at elevated temperatures and vacuum conditions is needed to remove water/organic solvent mixtures after fraction collection. In SFC-based purification, $CO_2$ evaporates immediately upon sample collection, generating much smaller fractions in pure methanol. Evaporation can be completed within a few hours, significantly shortening the purification cycle time. To obtain a comprehensive structure−activity relationship, libraries in lead optimization programs often include compounds that are acid/base-labile. Compromise between these compounds' stability and chromatographic behavior has frequently raised issues in RP-HPLC-based purification. SFC-based purification becomes particularly useful for these types of compounds because it does not necessarily require a mobile phase additive. In addition, SFC-based purification eliminates issues associated with trifluoroacetic acid (TFA) salts that are often seen in RP-HPLC-based purification, such as the difficulty in assessing the molar ratio between the TFA and the target compound during salt formation.

Figure 12 shows an example of a library compound purified by the preparative SFC/MS system. This specific compound library contains acid/base-labile functional groups. Tests on RP-HPLC systems without any mobile phase additive yielded split peaks and poor separation. When purified on the SFC system, the peak shape was well-defined. With a shallow gradient, we were able to achieve baseline separation between the product of interest and close-eluting impurities.

As expected, mass-directed purification is also particularly useful for compounds with low UV absorbance. An example is shown in Figure 13. Erythromycin, a natural-product-like compound, has a low UV response but fairly good ionization efficiency in electrospray positive mode due to a tertiary amine group. Collections based on MS response, in this type of case, would become very useful in assuring a successful outcome. This example also highlights the versatility of the SFC system for the separation of not only synthetic compounds but natural products, as well.

The preparative SFC/MS system has been primarily operated in mass-directed collection mode. We would like to point out that alternative collection approaches, such as UV threshold or fixed-time window, can also be used without the need for instrument modification.

## Conclusion

We have developed a preparative SFC/MS system that allows real-time fractionation based on an MS signal. Issues regarding the software integration and the SFC/MS interface have been resolved. A second UV detector, located after the fraction collector, was used in assessing the delay time and monitoring collection in real time. Column efficiency was optimized through flow path modification. Use of a wide bore collection needle was found to significantly reduce the linear flow rate at the collector and minimize aerosol formation. Overall recoveries were >85% at flow rates up to 30 mL/min. Generally good peak shape and column loadability were achieved with 2-ethylpyridine columns without any mobile phase additive. This system has been used successfully in library purification as a tool that is complementary to our RP-HPLC/MS-based purification platform.

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