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SFC/MS in drug discovery at Pfizer, La Jolla

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Abstract

We report the use of supercritical fluid chromatography/mass spectrometry (SFC/MS) for numerous applications in drug discovery at Pfizer, La Jolla. Namely, SFC/MS has been heavily relied upon for analysis and purification of a diverse set of compounds from the in-house chemical library. Supporting high-speed SFC/MS quality control of the purified compounds is made possible at high flow rate SFC along with time-of-flight mass detection. The flexibility of SFC/MS systems has been extended with the integration of an atmospheric pressure photoionization source (APPI) for use with more non-polar compounds and enhancements in signal to noise. Further SFC/MS applications of note include chiral analysis for purification and assessment of enantiomers and SFC/MS analysis of difficult to separate hydrophobic peptides.
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1. Introduction

Supercritical fluid chromatography coupled with mass spectrometry (SFC/MS) was first reported in 1978 by Randall and Wahrhaftig and the first analysis of pharmaceuticals using packed column SFC/MS was reported in 1985 by Crowther and Henion [1,2]. Between 1985 and 1997, the science progressed at a moderate pace and is summarized in an excellent review by Combs [3]. Since then, packed column SFC/MS has progressed from being a unique tool for analyzing specific problems on custom systems, to a general use analytical tool in the pharmaceutical industry.

A current review of SFC/MS technologies could fill this edition, so in order to limit the scope of this paper, we will discuss only applications from our labs at Pfizer in La Jolla. Within the drug discovery process, SFC/MS has made contributions from the analysis of crude combinatorial library mixtures, including development of ultra-fast SFC/MS for post-purification analysis, to chiral analysis for medicinal chemistry.

We have coupled SFC with atmospheric pressure photoionization (APPI) mass spectrometry as an alternative method for library analysis and analysis of difficult non-polar compounds. Application of SFC/MS/MS for use in ADME analysis has been previously reported by Hoke et al., although such work is not presented in this review [4,5]. Lastly, we have used SFC/ESI-MS for the analysis of peptides and cytochrome C (both the whole protein and a digest) to extend its application to separation/detection of hydrophobic compounds.

LC/MS is used extensively in drug discovery for compound purification, purity assessment, compound identification, pharmacokinetic studies, and a variety of proteomics applications, to name a few. However, in such an exhaustive use of LC/MS, some of its limitations, including the speed of LC separation and its application to highly hydrophobic compounds have been noted. In pharmaceutical drug discovery, where large libraries of chemically diverse compounds

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are generated and analyzed, the means to complement or improve upon LC/MS analysis is invaluable.

Supercritical fluid chromatography offers an excellent alternative/complement to reverse-phase LC analysis. As a normal phase technique, separation of highly hydrophobic species is possible while the increased retention of polar compounds can also be useful. Normal phase HPLC is not ideally suited for high-throughput or to handle compound diversity because of long run times, retention inconsistencies caused by slight changes in mobile phase solvent, and incompatibility with trace levels of water present in many solvents. Also, mobile phase gradients are not recommended due to the irreproducibility of the separations. Alternatively, the speed and durability of packed column SFC has been shown to handle large sample quantities, reproducibly, in shorter time [6,7]. For diverse compound libraries, a polar mobile phase modifier with and without additives extends the SFC polarity window to include organic acids and bases [8–10].

The coupling of SFC to MS produces a robust and flexible instrument highly suited for a number of drug discovery applications. Because the SFC eluent rapidly expands, thus assisting nebulization, when leaving the end of a capillary, SFC is actually more amenable to electrospray, APPI, and APCI MS source integration than HPLC [11]. In the cases of APCI and APPI, where the ideal analytes often tend to be more non-polar, SFC is a perfect chromatographic companion.

2. Methods

2.1. General instrumentation

Two analytical instrument configurations were employed for all SFC/MS experiments presented. Initial purity assessment by SFC/MS prior to preparative scale-up was performed on a system composed of an Agilent 1100 LC/MSD (Agilent, Palo Alto, CA), a Berger Supercritical Fluid Chromatograph (Berger Instruments, Newark, DE), and a Leap HTS PAL autosampler (Leap Technologies Inc., Carrboro, NC). Two computers were employed to control this system. The Leap HTS PAL autosampler was controlled by Cycle Composer software™ and the Agilent MSD by Chemstation running on a single computer. The Berger SFC was controlled by Berger Chemstation on a separate PC. Synchronization was achieved using a combination of contact closure signals and custom interfacing software. Automated data analysis was completed using a highly customized version of Agilent's GC/MS Chemstation software on an offline computer. The Agilent MSD was used with APCI, ESI, and APPI ionization sources.

For subsequent ultra-fast, peptide, and chiral work, the Agilent MSD was replaced with a Micromass LCT time-of-flight mass spectrometer (Micromass, Manchester, UK) and the Berger SFC system was replaced by a Jasco 1580 SFC system (Easton, MD). The Jasco SFC system, the HTS PAL autosampler, and the Micromass LCT were all operated within the Micromass MassLynx™ 3.5 software. The Micromass LCT also had APCI, ESI, and APPI ionization source capability.

All LC/MS data presented was done on an Agilent 1100 LC/MSD system with a HTS PAL autosampler controlled by Chemstation, Cycle Composer, and custom synchronization programs.

2.2. High-throughput SFC/MS (library QC and pre-preparative analysis)

Because libraries can contain highly diverse chemical entities with a varying degree of polarity, we developed a single LC/MS method that provided adequate separations of a diverse set of standards. The routine LC/MS method used a Peeke Scientific Hi-Q, 5 μm, 4.6 mm × 50 mm column with the Agilent 1100 LC/MSD system (Agilent Technologies, Palo Alto, CA). The LC column temperature was held at 35 °C. Flow rate was 2.25 ml/min with a 1/1 split into the ELSD/mass spectrometer. The mobile phase gradient consisted of a 3-min ramp from 100% water to 100% acetonitrile (each buffered with 0.1% acetic acid). The total LC/MS cycle time (injection-to-injection) was 5.1 min.

The chromatographic conditions for the Berger SFC system employed a 4.6 mm × 150 mm Zymor Zyrosil–Pegasus (Zymor, Wayne, NJ) packed column with the 5 μm, 60 Å pore size particles. The column oven was maintained at a temperature of 35 °C. The mobile phase utilized CO_2 with a methanol gradient from 5 to 60% at 18% per min. The flow rate for the analysis was 5.6 ml/min and the outlet back-pressure was maintained at 140 bar. Sample injections were made by a HTS PAL autosampler equipped with a 10 ml sample loop. Total cycle time (injection-to-injection) was 3.75 min.

2.3. Ultra-fast SFC/MS

Ultra-fast SFC separations with a total cycle time of 40 s (injection-to-injection) were performed using a Jasco 1580 SFC system with a mobile phase flow rate of 10 ml/min. The Jasco SFC system was operated from within the Micromass MassLynx software, which also controlled the LCT/TOF mass spectrometer and LEAP autosampler. The LEAP autosampler was modified to incorporate a valve self wash module to provide a fast (10 s), independent methanol rinse of the autosampler injector amenable to the ultra-fast method. The 5–60% methanol in CO_2 gradient was performed over a 30 s ramp time. System backpressure was regulated at 130 bar. A 30 mm × 4.6 mm Zymor Zyrosil–Hybrid® stationary phase chromatographic column with 3 μm particles and 60 Å pore size was used for all ultra-fast methods.

2.4. Chiral SFC/MS

All chiral analyses were performed with Chiral Technologies (Exton, PA) columns. Chiefly, the ChiralPak AD–H

column, 4.6 mm × 250 mm, 5 μm particle, was used for analytical runs due to its improved resolution over the standard 10 μm particle. Chiral methods were developed from isocratic runs of CO_2 with varied percentages of alcohol modifiers (methanol, ethanol, isopropanol). Operating flow rates from 0.5 to 4.0 ml/min were evaluated, however flow rates above 2 ml/min were not ideal for preparative scale-up due to hardware limitations. Translating the column diameters from 4.6 to 21 mm i.d. is roughly a factor of 20. While the preparative SFC pumps are capable of delivering flow rates up to 200 ml/min, the system is not designed to sustain flow rates in excess of 60–70 mL/min for high-throughput applications. At those flow rates, the evaporator/trimmer heater assemblies are operating at maximum capacity and thus fail rapidly. Therefore, imposing analytical flow rate limitations of <3.5 mL/min is warranted.

2.5. SFC/APPI-MS

SFC/APPI-MS has been performed on both SFC/MS systems previously described. The Agilent system used the PhotoMate APPI source developed by Agilent and Syagen (Tustin, CA). Syagen built a custom APPI source for our Micromass LCT system.

2.6. Peptide and protein SFC/MS

SFC grade CO_2 was modified with methanol containing a mixture of water, ammonium acetate, acetic acid, and isopropylamine (Modifier #8) and run in a gradient from 10 to 50% over 5 min. Flow rates from 1.0 to 3.0 ml/min with outlet pressure held at 150 bar were tested. 4.6 mm × 50 mm Phenomenex Luna 5 μm Cyano and Metachem Diol and Ether columns were used. Various other column temperatures and flow rates were also investigated, but did not perform as well. For analytical runs, 4 μl of 0.02 mg/ml solutions of gramicidin were injected. All preparative SFC was performed on the Berger automated prep SFC. A Zyrosil CN/RP (21.2 mm × 150 mm, 5 μm, 100 Å) column was used with a flow rate of 50 ml/min, gradient of methanol from 30 to 60% over 10 min, backpressure of 110 bar and column temperature of 60 °C.

3. Results/discussion

3.1. High throughput SFC/MS (library QC and pre-preparative analysis)

SFC/MS has been exploited for the purpose of high-throughput QC analysis of large numbers of samples such as combinatorial libraries [12,13]. Packed column SFC/MS is particularly suited to this purpose based on the ability to perform reproducible separations at a rapid rate with fast column re-equilibration. APCI has been coupled with SFC for over ten years and is an excellent ionization source for use with SFC [11,14–17]. The interface to the APCI source used in our laboratory employs a split from the high-pressure mobile phase downstream of the UV flow cell. The high linear velocity of the SFC mobile phase results in minimal delay between UV and MS detector signals. The APCI source is particularly suited to high flow rate effluents (to 10 ml/min) from fast SFC gradients unifying effective compound identification with rapid SFC separation. Both quadrupole and API-TOF mass spectrometers have been used in our laboratory for this application.

The system described above was also used to provide critical data needed prior to preparative-SFC purification. Preparative-SFC exhibits advantages over HPLC in many respects, and its application to combinatorial library purification has also been reported [18,19]. Often, high-throughput preparative-HPLC fails when samples are insoluble in water, elute in the void with sample solvents such as DMSO, or specific cases when impurities co-elute with the product. Limitations of high-throughput preparative-SFC include samples that are insoluble in methanol or CO_2, highly polar compounds, and similar to HPLC, cases where impurities co-elute with a given product.

The distinct advantages of preparative-SFC for high-throughput are numerous. Low viscosity and high diffusivity of supercritical fluids allow for efficient separations and fast column re-equilibration. The ability to change solvent strength by adjusting mobile phase composition, temperature, and pressure allow rapid method development. Because SFC is a normal phase chromatography technique, compounds that are not retained (more polar range of compounds) by reverse-phase HPLC often separate well using this complementary technique. Additionally, purification using CO_2 and methanol as the mobile phases dramatically decreases the drying time of fractions compared to acetonitrile/water/acid mobile phases used with standard high-throughput HPLC. Products often form salts when the LC mobile phase includes acids such as TFA, resulting in inaccurate weights/yields for the dried product. Removing acid from the mobile phase also reduces the risk of compound degradation. Finally, lower solvent and waste costs are realized when using SFC.

SFC has been utilized in our laboratory for high-throughput purification of over 120,000 wells yielding appropriate weight and high purity (>85% on two out of three detectors using SFC/MS/ELSD). The high-throughput preparative-SFC program relies on an accurate retention time correlation with analytical SFC/MS data. In so doing, we have realized a success rate for purifications above 90% with recoveries in excess of 90%.

Purity is determined from a post-preparative SFC/MS/ELSD analysis of an aliquot from each final product well. Fig. 1 gives a chromatographic illustration of one sample's composition from (a) crude analysis through (b) SFC purification analysis, to (c) final QC purity analysis of the product well by SFC/MS. This example illustrates a typical improvement in product purity, from 55% in the unpurified well to 96% after preparative-SFC.

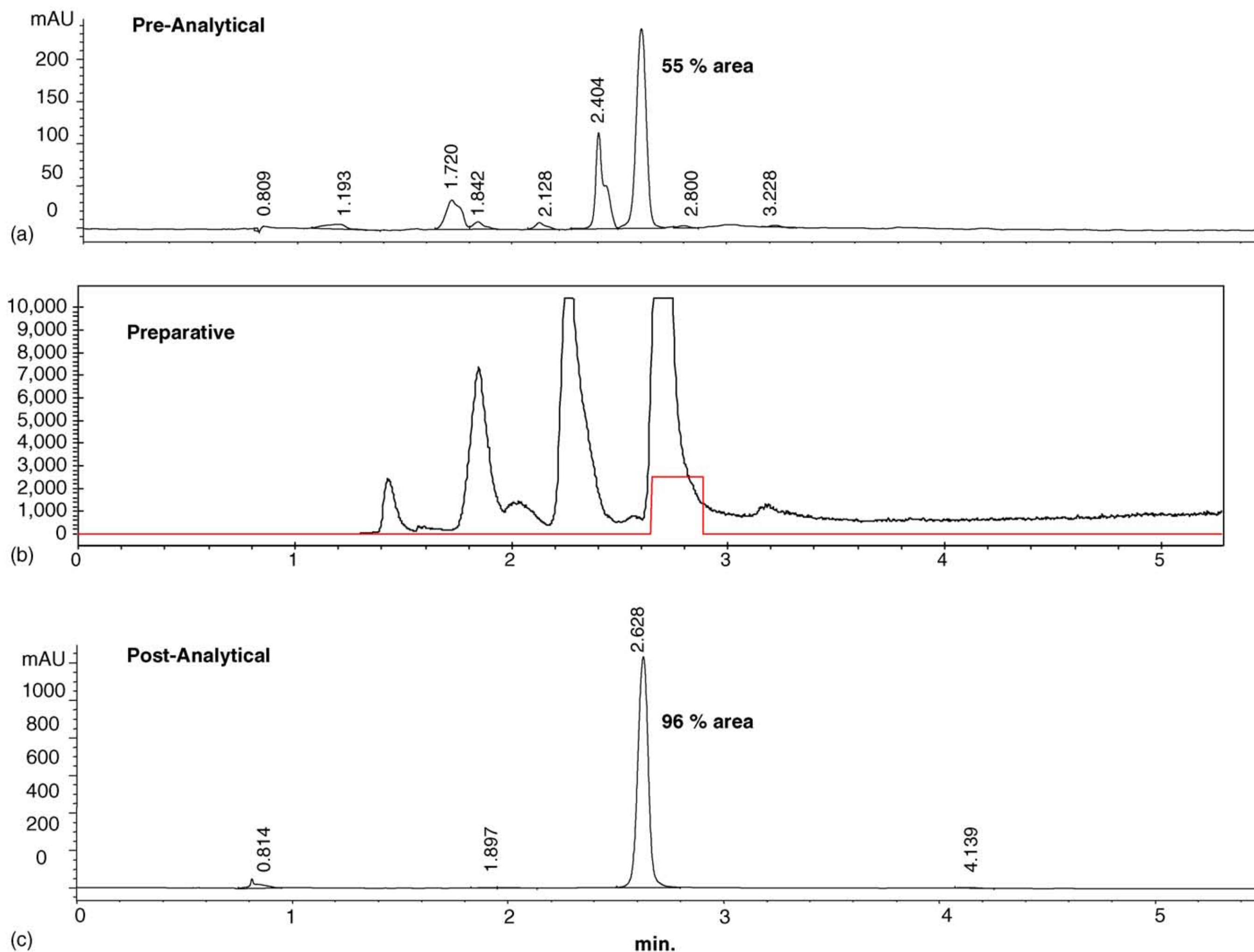


Fig. 1. Example of purity improvement through the preparative process based on SFC. (a) SFC/MS TIC analytical data of crude sample—identifies product and specifies retention time window. (b) SFC preparative run—rectangular trace represents time during which fraction collection occurred. (c) SFC/MS TIC chromatogram from purified well—product identified, integration yields 96% purity.

3.2. Ultra-fast SFC/MS

Shortening analysis time using SFC/MS maximizes instrument efficiency, which is critical in a high-throughput environment [5,9]. Advantages for a shorter method include a dramatic decrease in consumption of organic solvents and smaller data files for database storage. Additionally, according to the van Deemter curve, the optimal linear velocity is essentially flat for SFC. This means higher flow rates do not adversely affect the theoretical plates of the column, which is key for the ultra-fast SFC method [20]. The ability to operate at higher mobile phase flow rates (up to 10 mL/min) and still achieve reasonable chromatographic separations.

In order to effectively capture chromatographic data with such high-speed separations, time-of-flight mass spectrometers were incorporated to provide fast spectral sampling rates, <0.1 s, across a 100–1000 m/z range [21–23]. The ultra-fast SFC/MS method developed to support high-throughput sample QC used a mobile phase flow-rate of 10 mL/min and TOF sampling rate of 0.1 s/spectrum [24].

The standard high-throughput SFC/MS chromatograph discussed earlier consisted of a 2 min 40 s sample-to-sample injection cycle time was modified to dramatically reduce sample cycle time four-fold to 40 s. Table 1 shows a comparison

Table 1
Method parameters for 40 s cycle time 'ultra-fast' run and 'standard' 160 s SFC method

	Ultra-fast SFC (40 s cycle)	SFC (160 s cycle)
SFC run time (s)	30	144
Flow rate (ml/min)	10	5
Gradient (% MeOH)	5–60	5–60
Scan time (s)	0.05	0.1
Rinse injector	Valve self wash	Syringe
Mixer	250 μl	1 ml
Column (hybrid)	30 mm × 4.6 mm, 3 μm–60 Å	100 mm × 4.6 mm, 5 μm–100 Å

of the two methods' parameters. Since a diverse composite of samples were to be analyzed, a steep gradient of 5–60% methanol in CO_2 was used. Due to low mobile phase viscosity, SFC is amenable to higher optimal flow rates and smaller particle packed columns. The 4.6 mm × 50 mm, 5 μm analytical column was replaced with a shorter, 4.6 mm × 30 mm, smaller particle, 3 μm, packed column to allow shortened elution times while still providing sharp chromatographic peaks. As mentioned, the time-of-flight mass spectrometer was data-point optimized to record data at 0.1 s/spectrum to properly sample the narrow SFC peaks, which had baseline peak widths on the order of 1–3 s.

The ultra-fast SFC/MS analysis total-ion chromatograph is shown in Fig. 2 for a four-compound mixture (caffeine, sulfanilamide, pyridine, proprietary compound A) used to validate a wide range of compounds. With a four-fold decrease in total cycle time, the ultra-fast analysis baseline-resolved all four standards in less than 18 s, although the run continued 12 s more to ensure elution of potentially more polar species for general method applications. The retention time for all compounds was greatly shortened but because peak widths were also much narrower, the overall resolution was not decreased significantly. It should be noted that although the gradient runs were matched as well as possible, there was a measurable decrease in resolution for the first two standards, presumably due to the four-fold increased steepness of the methanol gradient employed in the ultra-fast method. However, the remaining standards showed less than a two-fold reduction in resolution from the previous method. Since post-purification SFC/MS QC analysis requires no correlation of retention times to preparative chromatographic systems, the ultra-fast method was best tailored to our laboratory's high-throughput purity analysis application.

An example of application of this method for post-purification SFC/MS analysis is demonstrated in Fig. 3. On relative total cycle time scale of each method, the ultra-fast total ion chromatogram run demonstrates comparable chromatography in a shorter period of time for verifying that the compound is purified. It should be noted that there was a measurable decrease in signal intensity from the ultra-fast run. Presumably, this sensitivity reduction may be attributed to the 0.05 s 'inter-scan' delay time required on the LCT TOF/MS system, which accounts for 50% of the detection duty cycle. However; for this application, where purified samples concentrations were expected to be on the order of 300 μM, sensitivity was not a concern.

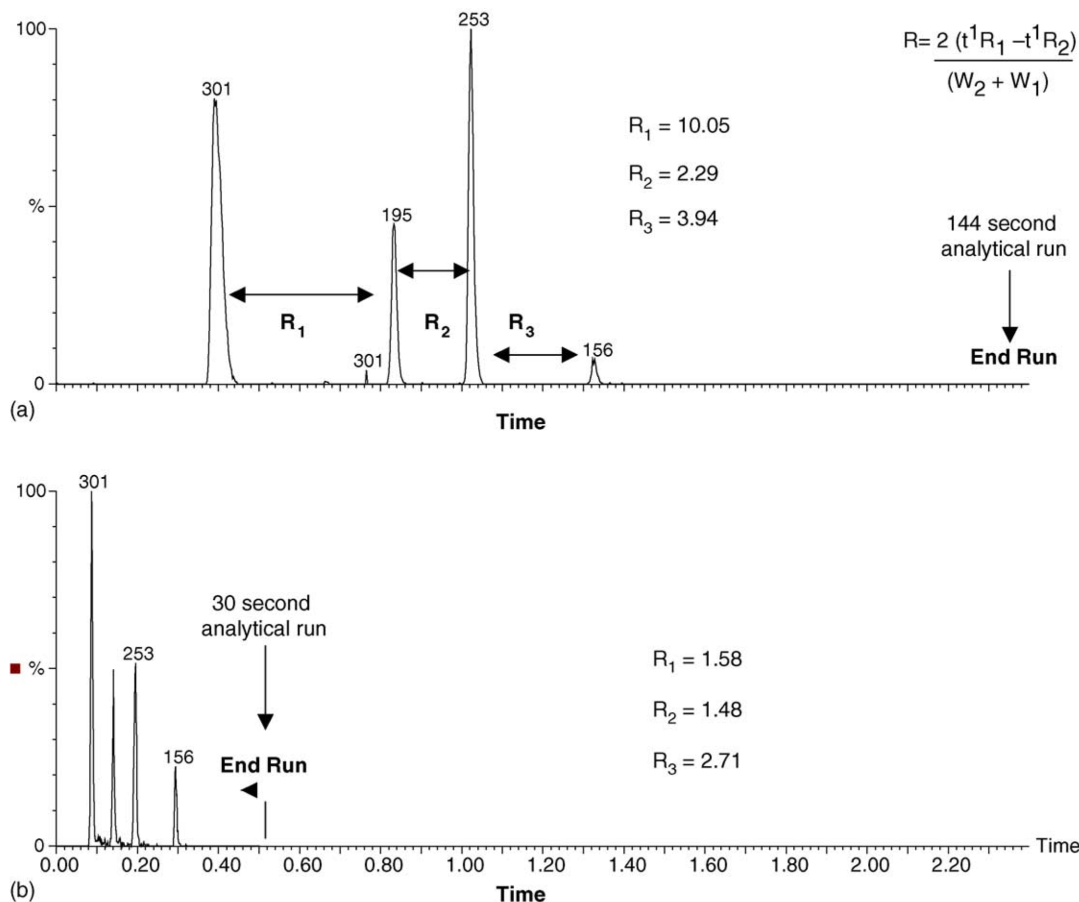


Fig. 2. Comparison of (a) traditional SFC method (144 s run time) and (b) ultra-fast run (30 s run time) on a four compound QC standard four-compound mixture (caffeine, pyridine, proprietary compound, sulfanilamide). For the ultra-fast run all compounds are baseline resolved in less than 20 s, but there is some loss of peak resolution due to a steep methanol modifier gradient.

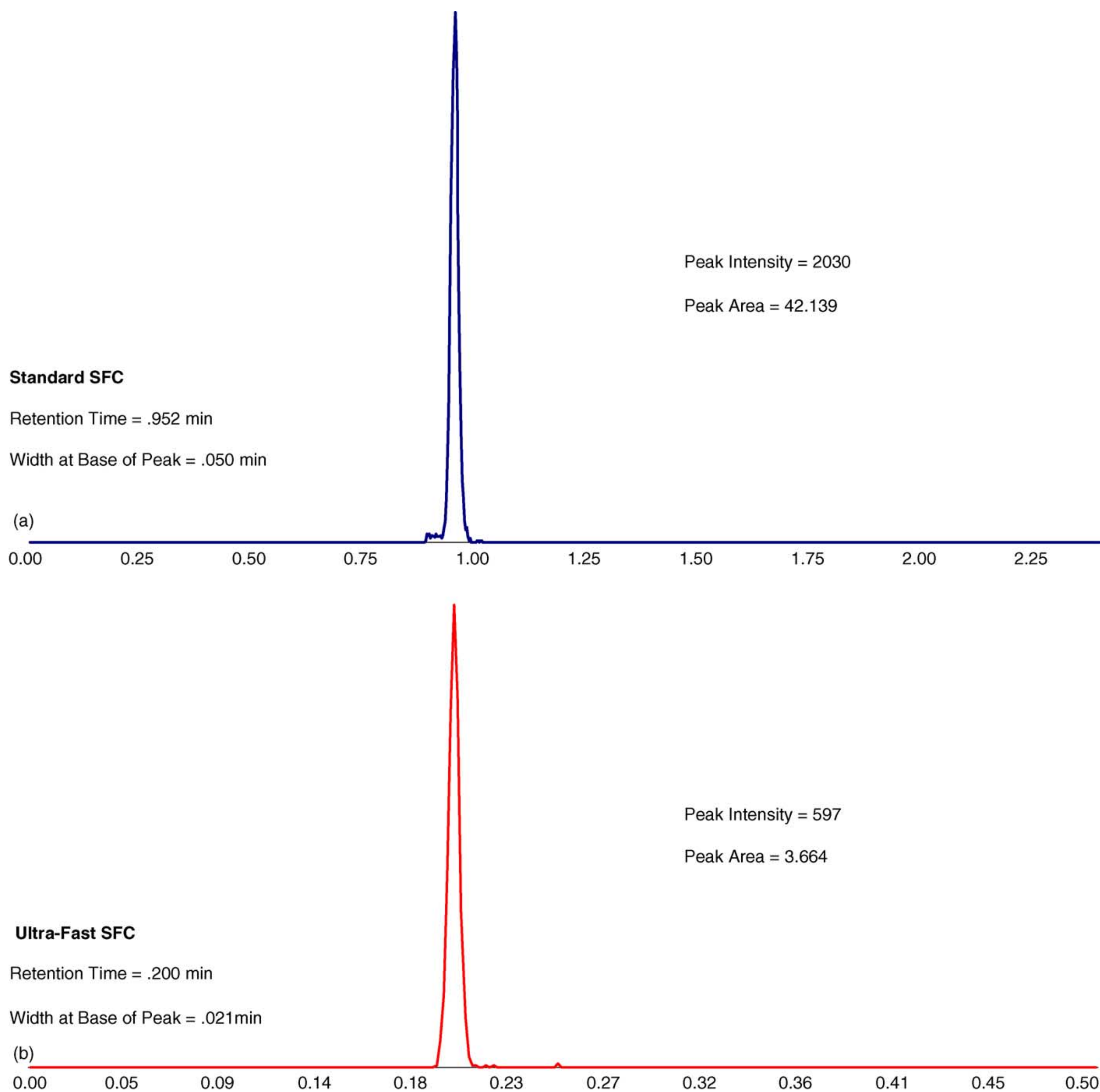


Fig. 3. Comparison SFC/MS chromatographic peaks of (a) standard and (b) ultra-fast SFC/MS analytical QC method for proprietary compound A.

3.3. Chiral SFC/MS

A large number of chemical entities for drug discovery contain chiral centers. More importantly, these enantiomers may differ in activity by orders of magnitude or worse yet, one enantiomer may be toxic. Quantitative assessment for potential purification of enantiomers is vital to compound characterization. SFC is ideal for chiral separations and has been used for this application for decades [25,26]. Typical LC separation of enantiomers require long chromatographic methods to resolve the compounds encompassing attempts on multiple columns. The increased separation efficiency and speed of SFC alleviates many of these woes [27–29]. Furthermore, the low temperature of the carbon dioxide mobile phase used with SFC is beneficial for chiral selectivity and compound stability [30].

Providing the different speeds of chiral separation is a great benefit for preparative scale purification. Baseline resolution of two enantiomers was demonstrated using APCI SFC/MS featuring a Chiral Technologies ChiralPak AD–H

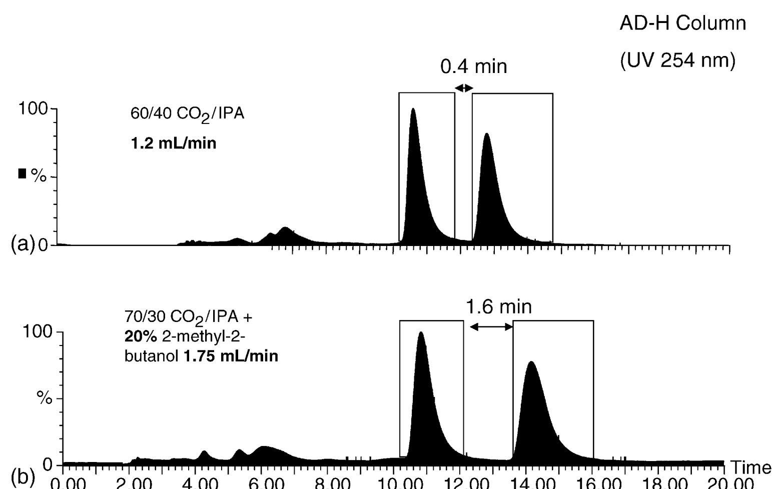


Fig. 4. SFC/MS analyses of two enantiomers using different conditions for pre-preparative runs. Separation (a) was best for multiple stacked injections for purification of bulk compound, while (b), with the addition of 20% tert-butanol to the mobile phase and change of composition and flow rate, provided better resolution for single shot purifications.

column, varying the CO_2/IPA mobile phase composition and flow rate (Fig. 4). If the goal were to purify bulk amounts (>1 kg) of each enantiomer, multiple injections would be necessary and stacked injections would be preferred as these yield potentially huge timesavings. In this case, maximum baseline resolution and optimum selectivity of the two enantiomers is critical, and the 60/40 CO_2/IPA mobile phase and 1.2 ml/min flow rate provided ideal separation method for stacked injection SFC purification. On the other hand, if maximum sample recovery and high enantiomeric purity from a single injection is necessitated, the addition of 20% tert-butanol to the methanol modifier along with changes in flow rate and mobile phase composition would result in greater separation of the peaks [31].

Accurately analyzing chiral compounds by SFC/MS is vital for properly assessing the potency of test ligands in biological assays. Shown in Fig. 5, two enantiomers, 404 *m/z*, were purified then later analyzed by TOF/MS with 260 nm UV detection. Although the UV signal indicates high purity of both chiral species, the SFC/MS total ion chromatograph revealed a significant impurities in addition to enantiomer B. Shown in Fig. 6, is the preparative run for a racemic mixture of another species of interest. Overall inhibition of this compound was found to be 1.2 μM, but specific enantiomer activity needed to be investigated. After SFC purification, analytical SFC/MS confirmed better than 99% purity of each enantiomer. The purified enantiomers were then re-submitted to biological screening revealing a dramatic difference in inhibition—one enantiomer had 15 μM inhibition, while the other 0.65 μM inhibition.

3.4. SFC/APPI-MS

While packed column SFC/MS originated with electron impact (EI) ionization and later expanded to CI, APCI, and ESI, recent successes have been reported coupling atmospheric pressure photoionization (APPI) with SFC/MS. [32,33] APPI, pioneered independently by Bruins and Syage, uses photons from a light source, typically krypton (10.0, 10.6 eV), to ionize analyte molecules.[34,35] Molecules can be ionized by various mechanisms with APPI. Direct photoionization occurs when the analyte undergoes a photon-induced loss of an electron to produce an $M^{\bullet+}$ ion. The ionization potential (IP) of the analyte must be of lower energy than the lamp source. $[M + H]^+$ can be produced when a photoionizable reagent (dopant) added to the mobile phase is photoionized and transfers a charge to the analyte or the M^+ ion abstracts a hydrogen from the solvent. Typical dopants include acetone and toluene (IP 9.71 and 8.82 eV, respectively). The ion species formed ($M^{\bullet+}$ versus $[M+H]^+$) depends on the proton affinity of the analyte, the solvent, and the inclusion of dopants.

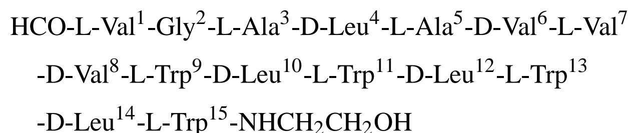
In many cases, APPI is more sensitive than ESI or APCI and has been shown to have extremely high signal-to-noise ratios with very little background (Fig. 7) [32,33,36,37]. SFC solvents and modifiers such as CO_2 and methanol (IP 13.79 and 10.85 eV, respectively) are not photoionized by the krypton lamp. This prevents secondary solvent–analyte reactions and enhances signal-to-noise ratios by minimizing the presence of background ions. Furthermore, APPI has been shown to be an excellent ionization source for SFC compatible analytes such as steroids, fat-soluble vitamins, PAHs, as well as other non-polar molecules including naphthalene and acridine [33,34,38–40]. Coupling SFC with APPI provides fast separation and high sensitivity for many compounds that are difficult to analyze with standard HPLC/MS techniques.

3.5. SFC/MS of peptides and proteins

Membrane proteins and their constituents are very attractive drug targets for pharmaceutical companies but also impose barriers when compared to soluble protein targets. Few

Fig. 5. SFC/MS analysis of two SFC purified enantiomers (404 *m/z*). Although enantiomer A was very pure, %e.e. > 97%, by both TIC and UV, enantiomer B was shown to have impurities that were detected only by MS.

crystal structures of membrane proteins are known, and despite the use of predictive protein structure software based on amino acid sequences very little is know about many of these important molecules. The study of these hydrophobic compounds has been hampered by the difficulty or inability to isolate these compounds. One approach to examining these proteins is to use small hydrophobic peptides as membrane models. Portions of the protein sequence can be identified by a variety of models that correspond to transmembrane helices [41]. Gramicidin is one such molecule that has been extensively studied in terms of function, structure, and gene regulation [42]. Gramicidin D is a mixture of membrane-spanning peptides which consists mainly of gramicidin A where position 1 may also be replaced by isoleucine in 5–20% of the molecules, gramicidin B where the tryptophan at 11 is replaced by phenylalanine, and gramicidin C where the tryptophan at 11 is replaced by tyrosine.



$$\text{HCO-L-Val}^{1}\text{-Gly}^{2}\text{-L-Ala}^{3}\text{-D-Leu}^{4}\text{-L-Ala}^{5}\text{-D-Val}^{6}\text{-L-Val}^{7}\text{-D-Val}^{8}\text{-L-Trp}^{9}\text{-D-Leu}^{10}\text{-L-Trp}^{11}\text{-D-Leu}^{12}\text{-L-Trp}^{13}\text{-D-Leu}^{14}\text{-L-Trp}^{15}\text{-NHCH}_2\text{CH}_2\text{OH}$$

Although synthesis of these compounds can be difficult, the complete purification often tends toward the impossible. Traditionally, peptides are separated using reverse-phase HPLC or analyzed by capillary electrophoresis when purification is not necessary. In the case of hydrophobic peptides, including many helical peptides, impurities often are difficult or impossible to resolve using standard HPLC techniques. RP-HPLC in combination with flash chromatography, hydrophilic interaction/cation-exchange chromatography,

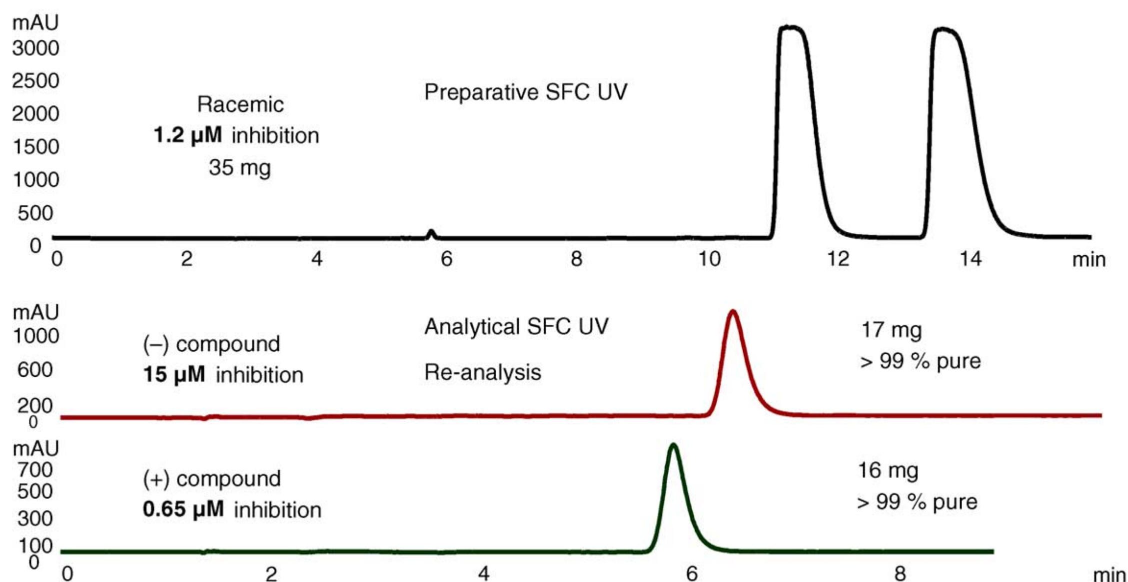


Fig. 6. Preparative scale purification of racemic mixture (1.2 μM inhibition) demonstrates a significant difference in inhibition activity, 0.65 μM vs. 15 μM, of each purified enantiomer.

micellar electrokinteic chromatography, and normal phase chromatography have all been used to analyze and/or purify these compounds [43–48]. Although good results can be obtained using these methods, usually multiple preparative steps or non-standard methods are required, lowering overall yield of purified materials. Additionally, analysis of hydrophobic peptides is often hampered by insolubility in the solvents used by the various chromatographic methods.

Even when chromatographic and solubility problems of these molecules are overcome, it can still be difficult task to determine their purity. The analysis of membrane proteins and hydrophobic peptides by electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI) mass spectrometry (MS) has been reported but is not in wide use [49–52]. Problems with MS analysis of these biomolecules include the same problems encountered in HPLC, such as solubility and solvent compatibility. In addition, the mass

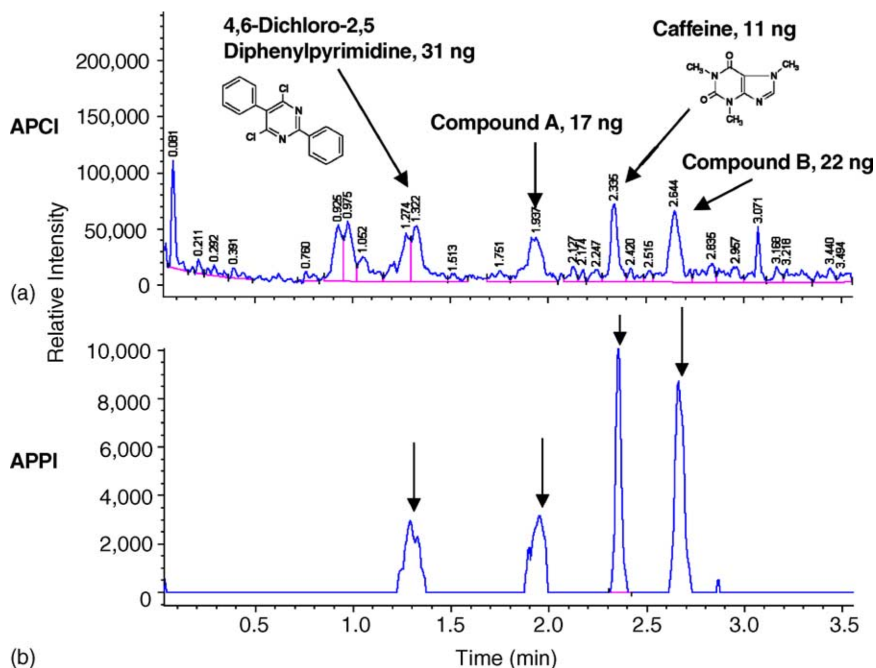


Fig. 7. SFC/MS analysis of standard test mixture using (a) APCI and (b) APPI on the Berger/Agilent system. All conditions were held constant except for ionization source.

spectral signal may be compromised when detergents are used since detergent carry-over can be a long lasting problem.

The analysis of peptides by SFC/ESI-MS has not been reported, most likely due to their extremely polar nature and SFC's historical incompatibility with these types of compounds. On the other hand as we have previously reported, the application of SFC/MS to diverse combinatorial libraries has been very successful at Pfizer as well as other companies [9,11,13,19,53]. The routine analysis of polar compounds by SFC/MS at Pfizer, including compounds containing highly basic amines as well as carboxylic acids, prompted further investigations. Success with these polar compounds inspired us to attempt to analyze hydrophobic peptides with SFC/MS. By using the proper combination of modifiers and columns, we demonstrated the rapid analysis of gramicidin and other peptides.

A commercially available sample of gramicidin D, a heterogeneous mixture of six components (Sigma, St. Louis, MO), was analyzed by both HPLC/MS and SFC/MS. The SFC/MS system separated the three forms of gramicidin (B, C, and A) in under 5 min whereas by HPLC/MS, it was difficult to achieve any notable separation of the three in less than 30 min [54]. We attempted multiple modifications of the methanol to increase the polarity range usable in the SFC system, using combinations of all or some of water, ammonium acetate, acetic acid, trifluroethanol (TFE) and isopropylamine. 'Modifier #8', methanol with 0.2% water, 0.01 M ammonium acetate, and 0.4% isopropylamine, was initially found to produce the best separation of the gramicidin, and later we found that methanol with 0.5% TFE worked as well (Fig. 8).

We then attempted to transfer this method to the preparative SFC system using the same mobile phase and a scaled up column. Using a gradient of 'Modifier #8' from 30 to 60% over 10 min, failed to separate gramicidin mixture. We then attempted to run the sample using the same conditions, but with pure methanol as the modifier to the CO_2, and achieved separation in under 6 min. One milliliter of a 2.24 mg/ml solution of crude gramicidin dissolved in methanol was injected onto the column. Center cuts of the gramicidin A and C peaks were collected, dried down, weighed, and re-analyzed by SFC/MS as described above. 1.49 mg of >95% pure gramicidin A was recovered and 0.45 mg of 85% pure gramicidin C were recovered.

Other peptides from 2 to 20 amino acids in length including oxytocin, leu and met enkephalin, angiotensin II, fragments of bradykinin and β amyloid, and a bovine cytochrome C tryptic digest were analyzed successfully by SFC/MS. Pictured in Fig. 9 is an SFC/MS analysis of Sigma HPLC peptide standard mix H2016. Mixtures of these and other peptides were resolved in less than 5 min including a cytochrome C tryptic digest (data not shown). Finally, full-length rabbit and bovine cytochrome C were analyzed using flow-injection analysis SFC/MS yielding the first known spectrum of a full length protein by SFC/MS (Fig. 10).

By modifying the supercritical fluid chromatography (SFC) solvent with a variety of polar additives, we have been able to exploit the improved speed, resolution, and normal phase properties of SFC for the separation of peptides with analysis by ESI-MS. Solubility problems encountered in RP-HPLC solvents are avoided since SFC is compatible with methanol, trifluoroethanol, chloroform, and many of the other

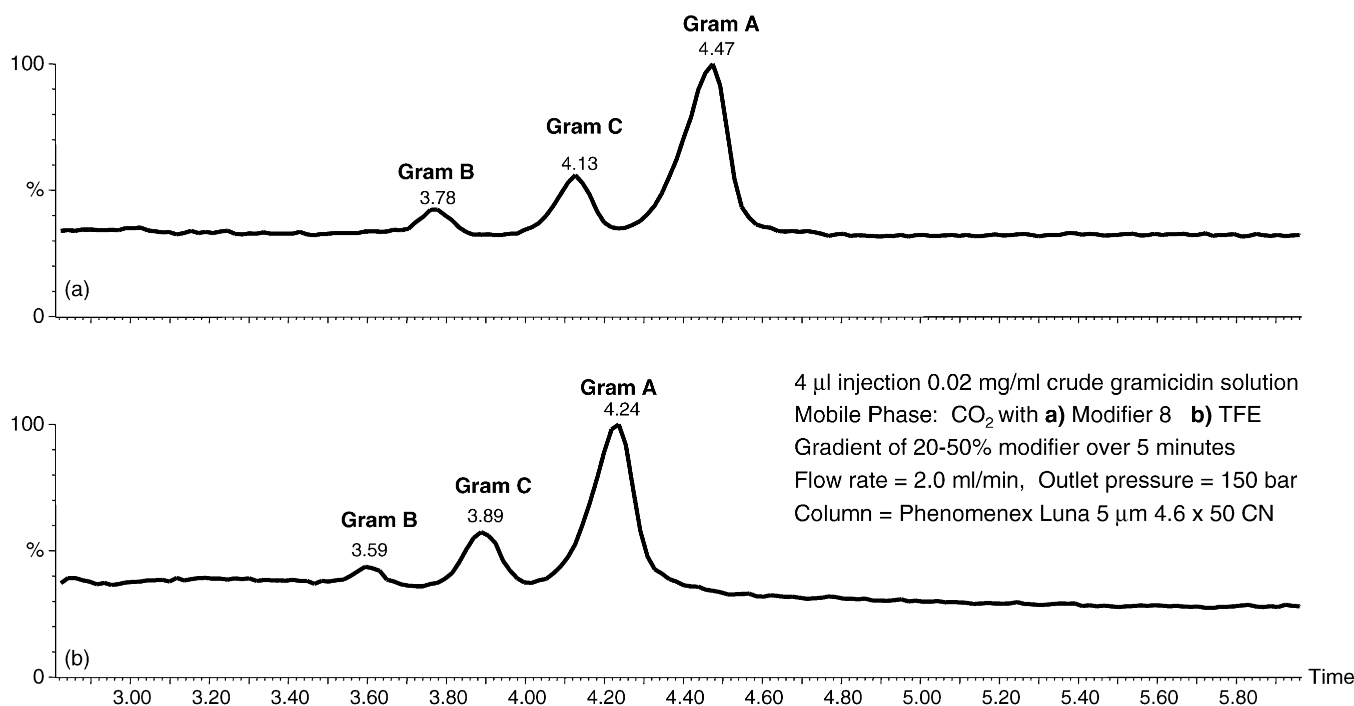


Fig. 8. SFC/ESI-MS of crude gramicidin mixture using CO_2 modified by (a) 'Modifier #8' and (b) trifluoroethanol.

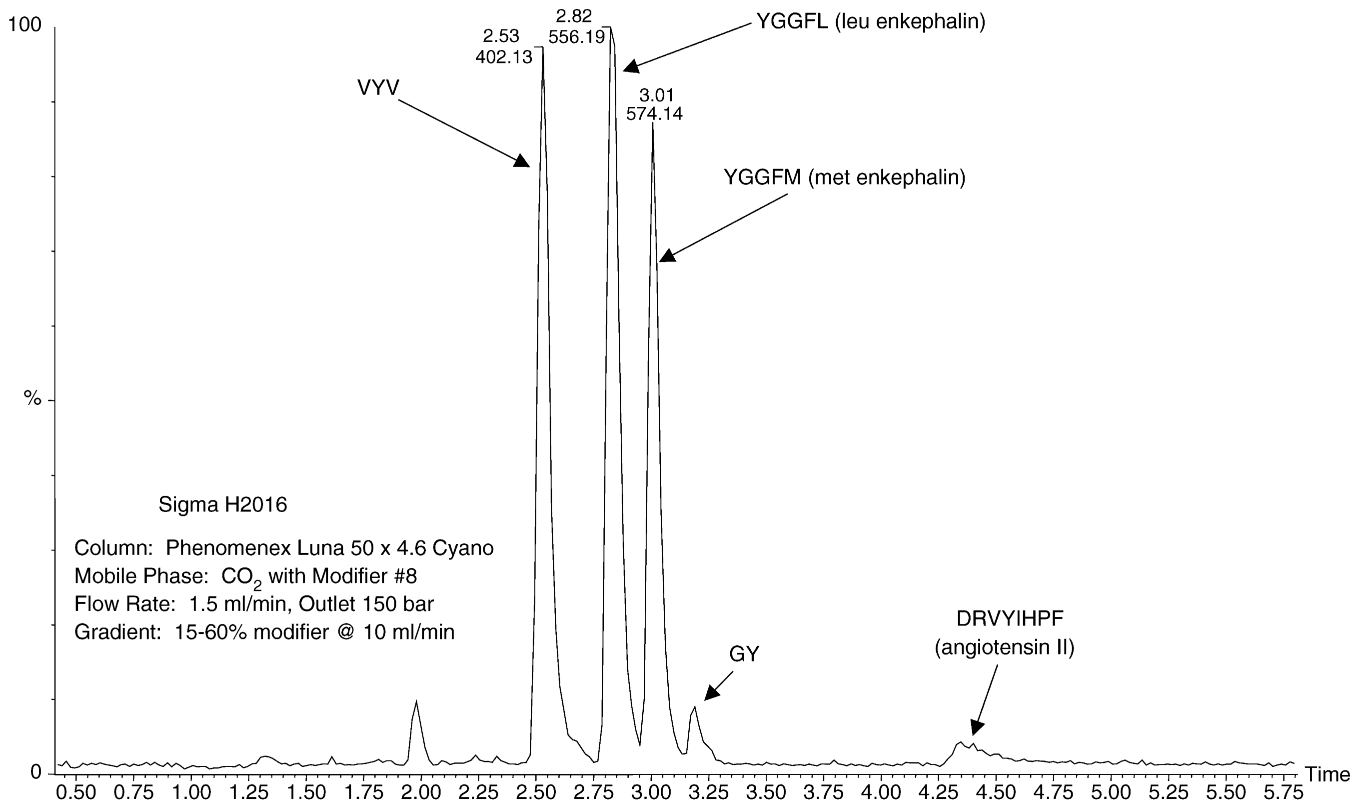


Fig. 9. SFC/ESI-MS of Sigma HPLC peptide standard mixture (H2016).

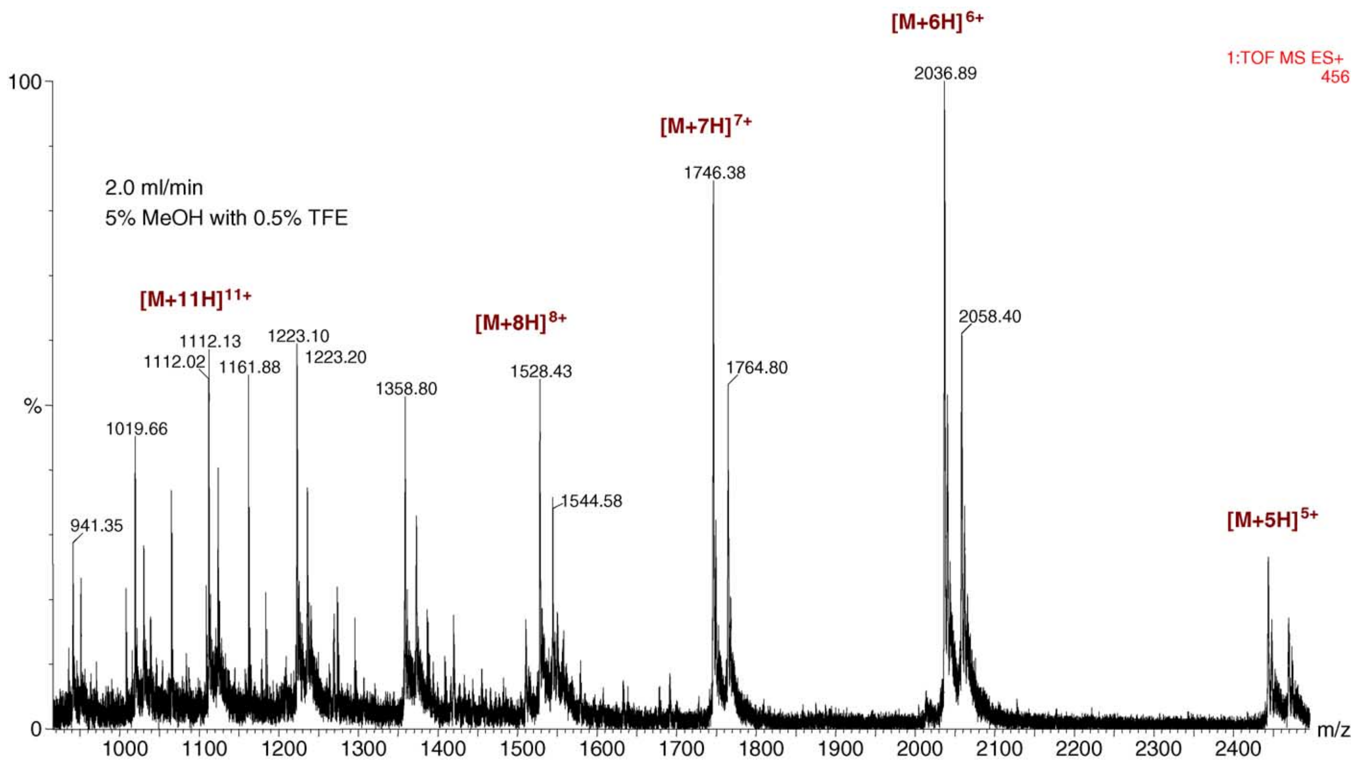


Fig. 10. Flow injection SFC/ESI-MS of cytochrome C (rabbit and bovine heart).

solvents used to dissolve hydrophobic peptides and proteins. The advances in speed and resolution along with simplicity of a single system make it an excellent method for these types of compounds.

4. Conclusions

From its infancy in 1978, packed column SFC/MS has grown from an interesting application of old technologies mated for specific studies, to a mature robust technique used for a variety of applications in drug discovery. In our labs, we have used SFC/MS for the high throughput analysis of chemical libraries over a period of four years. In that time, over one million compounds have been analyzed by SFC/MS and the original systems first put into use in 1998 are still operating. Because of the durability and throughput, the systems are used for both pre- and post-purification analysis and were an integral part of our high-throughput purification service.

Experimentation with the modifiers added to the CO_2 have enabled us to analyze a wider range of compounds than first imagined. Both proteins and peptides have been analyzed using SFC/ESI-MS, and gramicidin was purified by SFC based on methods developed on the SFC/MS systems. Chiral analysis, long a stronghold of SFC utility, consistently utilizes SFC/MS for both crude and final product analysis. Having multiple columns and solvents available have enabled us to solve every chiral problem we have been presented. While EI and APCI applications were the first reported, both ESI and APPI are now common ionization sources used in our labs with SFC/MS for problem solving.

Throughout the past decade, SFC/MS has experienced a huge leap in productivity, due to increase in reliability and robustness of both SFC and MS systems, the need to push drug discovery faster, and as a potential solution to old problems unsolved and new problems yet to be encountered. The availability of commercial systems will undoubtedly help this current boom continue. SFC/MS has proven to be a fast, robust, diverse, and most importantly, a useful tool for drug discovery.

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