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# Current Application of Mass Spectrometry to Combinatorial Chemistry

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**Review Contents**



During the past decade, combinatorial chemistry has become an integral component of nearly all drug discovery efforts (*1*−*4*). Mass spectrometry (MS) has played a key role in the advancement of combinatorial chemistry efforts: in reaction monitoring and optimization of library synthesis, in the purification of libraries synthesized in a parallel format, and in the assessment of library compound quality, as well as in screening for bioaffinity or pharmaceutical properties of the above. A number of useful books or book chapters (*5*−*8*) and review articles (*9*−*18*) have been published recently on the application of mass spectrometry to combinatorial chemistry.

A few words about the scope of this review article. As nearly all of the pharmaceutical industry now synthesizes and screens single pure entities generated in a parallel format as opposed to combinatorial mixtures (*19*), this review will deal primarily with parallel synthesis applications. Classical combinatorial libraries made with one-bead-one-compound or split synthesis methods are not reviewed here as efforts in this area are only minimally continued at present. For the interested reader, good recent reviews can be found encompassing the mixture library analysis by MS (*5*−*16*, *18*). Further, as the vast majority of combinatorial libraries currently being synthesized are small organic molecules (*20*), this review will not include efforts in peptide and oligonucleotide library synthesis. Relevant references on the analysis of oligomer libraries can be found in reviews (*5*−*16*, *18*).

Small-molecule libraries synthesized in the parallel format may be those directed toward either lead discovery or lead optimization and may be synthesized either on a solid support or in solution. The large numbers of discrete compounds generated require analysis and, most frequently purification. This review will focus upon how mass spectrometry assists in those efforts.

High-performance liquid chromatography (HPLC) is an important tool in the analysis and purification of discrete library compounds, often coupled with mass spectrometry (LC/MS). For the interested reader, a good review (*21*) of HPLC, including equipment, columns, and detectors, is available by LaCourse. A list of abbreviations used in this review may be found at the end of the article.

## MASS SPECTROMETRY IN LIBRARY ANALYSIS

Due to the high speed, high sensitivity, and specificity of detection, mass spectrometry is widely used in the analysis of combinatorial libraries. Atmospheric pressure ionization (API) techniques including electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are commonly used due to the ease of interfacing with liquid sample introduction and separation techniques. Research and development in the analysis of these libraries have been focused primarily on the improvement of throughput and automation in the analysis, interpretation, and reporting of data. At the same time, the use of one or more auxiliary detection techniques such as ultraviolet (UV) or evaporative light scattering detector (ELSD) in addition to mass spectrometry allows for better assessment of the purity of library compounds.

**FIA/MS.** Flow injection analysis (FIA) is the simplest form of introducing samples into mass spectrometry, and it has been widely used in the analysis of combinatorial library samples. This technique offers the highest throughput, ease of use, and ease of automation. Richmond and Goerlach (*22*−*24*) reported methods of minimizing sample carryovers in FIA/MS analysis of combinatorial libraries. This was done by sorting the samples before the analysis to maximize the molecular weight difference between samples in the analysis queue and to minimize the opportunity that consecutively measured wells contain samples with similar building blocks. Cycle times less than 1 min were reported with a carryover of 0.01%. A software application was developed to automatically report the sample purity and calculate sample carryover by an automatic spectrum comparison method (*25*, *26*). A quasi-molecular ion discovery feature was also implemented (*27*) in the automated data processing program. Automated FIA/MS analysis and reporting were also used in the analysis of fractions from the purification of combinatorial libraries (*28*). Whalen et

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al. developed software to allow automated FIA/MS analysis from 96-well plates (*29*). The system optimizes the interface for MS and tandem mass spectrometry (MS/MS) conditions and reports the results in an unattended fashion.

**High-Throughput FIA/MS.** One important advantage of FIA/MS analysis is its high throughput. Samples are analyzed routinely with less than 1 min/injection with a single injector. Higher throughput is typically achieved by parallel injection from multiple injectors when injector washing becomes rate limiting. Wang et al. (*30*) used a parallel eight-probe injector system to achieve an effective throughput of 7.5 s/sample. The method was used for the analysis of combinatorial libraries synthesized in microtiter plates and compounds purified in deep well plates. Morand et al. (*31*) reported an improved method of parallel sampling and serial injection of samples using an eight-probe injector system from microtiter plates with a throughput of 4 s/sample. Improved analysis rates were achieved without sacrificing the integrity of the flow injection peak profile as baseline resolution was maintained for all samples.

A different approach to increasing the throughput of sample introduction into a mass spectrometer is to use a multichannel device. A device with an array of electrospray tips was developed by Liu et al. (*32*) for FIA/MS analysis. The device uses an independent electrospray exit port for each sample to minimize sample cross-contamination and increase throughput. Their results demonstrated a throughput of 5 s/sample. An alternative multichannel system was developed based upon a subatmospheric ESI interface that couples 96-well plates directly with an ESI interface and that enabled analysis of samples at a speed of 10 s/sample with a 120-nL sample consumption (*33*). The infusion system was used in the analysis of preparative HPLC fractions from a library synthesis.

**Reaction Monitoring and Optimization.** FIA/MS has been used in reaction monitoring and in optimization involving detection of enantiomers of stereospecific reaction. Reetz et al. (*34*) prepared isotopically labeled pseudoenantiomers and pseudoprochiral compounds, which behave chemically as a racemate or as a meso compound. Kinetic resolution of a racemate gives two products with different molecular weights, enabling the ratio to be determined using mass spectrometry. The authors used FIA/MS with a microplate autosampler to allow analysis of up to 1000 reactions/day. Guo et al. (*35*) reported an alternative mass-tagging approach to the measurement of enantiomeric excess by FIA/MS. In their method, an equimolar mixture of pseudoenantiomeric mass-tagged pairs of reagents was prepared that differ in a substituent remote to the chiral center. Szewczyk et al. developed a method to monitor the progress and to optimize reaction by quantitatively converting the product ketones, which are invisible in ESI detection into ESI-active derivatives (*36*). The method was used to monitor three experiments involving 33 different substrates with more than 170 determinations of yield. McKeown et al. incorporated into the synthesis of combinatorial libraries MS sensitive linkers (*37*). Upon photochemical cleavage after synthesis, these linkers can be monitored by high-throughput FIA/MS to provide information about the yield of the synthesis and the photocleavage processes.

**LC/MS.** Despite its speed and ease of use, FIA/MS results can be affected by ionization suppression due to unresolved components and impurities in the sample. The use of short columns, high flow rates, and generic elution gradient HPLC methods provide purification and separation of library compounds before detection with mass spectrometry while maintaining a reasonable throughput. Such fast LC/MS techniques have become a crucial enabling technology in the analysis of combinatorial libraries from parallel synthesis.

**Optimization of Fast HPLC and LC/MS.** Weller et al. (*38*) developed generic, high-flow, reversed-phase gradient HPLC methods for application in the analysis and purification of parallel synthesis libraries. The methods enable analysis of over 300 compounds/day or purification of up to 200 compounds/day on a single system. Hardware and software modifications allowed continuous unattended use for maximum efficiency and throughput. Mutton (*39*) described fast HPLC methods with short (20−100 mm) columns swept by fast yet shallow gradients and compared the results with those obtained with 150-mm columns and slow gradients. The resolution losses incurred with shorter columns were minimized by employing fast flow rates. High-quality performance was obtained with turnaround times of 5−10 min. An overall 5-fold enhancement in the rate of information generation was obtained. Goetzinger and Kyranos (*40*) investigated different packing materials, gradient methods, and sample solvents for the development of ultrafast gradient HPLC methods. Using commercially available equipment and short columns (<30 mm) packed with small particles (<4 $\mu$m), a 1-min total analysis time was achieved with a peak capacity of 49. These methods were used for quality control of spatially addressable combinatorial libraries. Pereira et al. (*41*) investigated different buffer types and column geometry effects in high-throughput LC/MS. All the tested buffers, phosphate, acetate, and acetic acid, exhibited good resolution while the separation time varied from 4 to 12 min, with acetic acid giving the shortest separation time. By shortening the column length and increasing the flow rate, the separation time was reduced with no change in selectivity.

Fast LC/MS methods have been used to assess library quantity and purity as well as to triage purification of compounds. Zeng et al. (*42*) developed one of the first fully automated analytical/preparative LC/MS systems for the characterization and purification of compound libraries derived by parallel synthesis. The system incorporated fast, reversed-phase LC/ESI-MS analysis (5−10 min). Postdata acquisition purity assessment of compound libraries was performed automatically using software control. Compounds falling below a threshold level of purity were automatically purified with HPLC. The real-time purity assessment eliminated the need for postpurification analysis or pooling of fractions collected.

With the widespread application of combinatorial chemistry in drug discovery, there are increasing numbers of compounds being tested for pharmaceutical profiling (absorption, distribution, metabolism, excretion, and toxicity or ADME/tox). The need for high-throughput analysis of these compounds stimulated active research in the improvement of LC/MS-based quantitation techniques. Wu et al. (*43*) investigated monolithic columns for high-throughput bioanalysis application. Due to the lower pressure drop on a monolithic column than on a particulate column, a high flow rate (6 mL/min) was used for a 4.6 × 50 mm monolithic column. The separation efficiency and signal/noise ratios for this separation remained almost constant at flow rates of 1, 3, and 6 mL/

min, respectively. The chromatographic retention time, separation quality, peak response, and sensitivity were highly reproducible throughout a run of 600 plasma extracts. Romanyshyn and Tiller (*44*) examined the effects of column length and gradient time on ultrafast chromatographic resolution. By judicious adjustment of column length and gradient slope, chromatographic integrity of chemically diverse analytes was maintained at much faster elution speed. The optimized method development strategy enabled separations on 2 × 20 mm HPLC columns at flow rates of 1.5−2 mL/min with full linear gradients achieved in 1 min. Cheng et al. (*45*) described a simple and comprehensive LC/MS/MS strategy for the rapid analysis of a wide range of pharmaceutical compounds. The authors started with a column that provided a good peak capacity at short gradient run times and then employed high flow rates to achieve a good gradient peak capacity. Using this fast LC/MS/MS method, it was possible to separate and identify a wide range of analytes in 1-min gradient analyses. Zhang et al. (*46*) described an isocratic LC/ESI-TOF-MS method for quantitation and accurate mass measurement of five tricyclic amine drugs fortified in human plasma with a per sample run time of 18 s using a short $C_{18}$ column (15 × 2.1 mm i.d.). The authors used a highly aqueous mobile phase at a flow rate of 1.4 mL/min. Samples were prepared by off-line liquid−liquid extraction. An acquisition speed of 0.2 s/spectrum accommodates these fast separation conditions. Accurate masses were determined by two-point internal mass calibration with postcolumn addition of standard. Results showed a mass error not greater than 9 ppm for all the target compounds. Zweigenbaum and Henion (*47*) demonstrated more than 2000 samples in 24 h with LC/MS/MS separation and quantitation for compounds in control human plasma. The method includes sample preparation with liquid−liquid extraction in the 96-well format, an LC separation of the five compounds in less than 30 s. Hsieh et al. (*48*) developed a direct injection bioanalytical method based on a single column LC/MS/MS for pharmacokinetic analysis. After mixing with a working solution containing an internal standard, each plasma sample was directly injected into a polymer-coated mixed-function column for sample cleanup, enrichment, and chromatographic separation. The stationary phase incorporates both hydrophilic and hydrophobic groups, which allow proteins and macromolecules to pass through the column due to restricted access to the surface of the packing while the drug molecules are retained on the bonded hydrophobic phase. The analytes retained in the column were eluted with a strong organic mobile phase. The total analysis time was 5 min/sample. Yu and Balogh (*49*) developed a fast LC/MS method and compared it with an FIA/MS method for effective quantitation. By applying fundamental concepts of fast LC, such as using a small column (30 mm × 2.1 mm, 2.6-$\mu$m particle) at an elevated temperature (40 °C), and a higher flow rate (1.0 mL/min), they were able to reduce the LC cycle time from more than 20 to 2.7 min. The authors compared the limits of detection and quantitation, linearity, precision, and accuracy for each analyte. The results indicate that fast LC/MS is generally better than FIA/MS analysis. Romanyshyn et al. (*50*) developed an LC/MS/MS method for quantitation using rapid ("ballistic") gradients on narrow-bore, short HPLC columns and compared the fast gradient approach with the more traditional high-organic isocratic LC/MS/MS methods. Fast isocratic methods frequently elute the analytes of interest at the solvent front, the region of unretained salts. The fast gradient method, in contrast, retains analytes on-column until well after the solvent front has eluted. Overall sample throughput is increased with fast gradient methods due to reduced analytical run time, decreased method development time, and fewer repeat analyses. Onorato et al. (*51*) used a multiprobe autosampler for parallel sample injection, short, small-bore columns, high flow rates, and elevated HPLC column temperatures to perform LC separations of idoxifene and its metabolite at 10 s/sample. Sample preparation employed liquid−liquid extraction in the 96-well format. An average run time of 23 s/sample was achieved for human clinical plasma samples.

**LC/MS and Hyphenated Techniques for the Assessment of Library Purity.** Frequently one or more auxiliary detection methods, such as UV, nuclear magnetic resonance (NMR), ELSD, and more recently chemiluminescent nitrogen detector (CLND), are combined with LC/MS to provide a better overall assessment of library purity. These different detection methods often provide complementary capability in detection selectivity, range of sensitivity, and linearity of response.

Kibbey (*52*) compared HPLC quantitation of combinatorial libraries with ELSD and UV detection. The ELSD detector's response is structurally independent and requires no chromophore, which makes it well-suited to HPLC analyses of mixtures of dissimilar compounds. Furthermore, the ELSD exhibits a nearly equivalent response to compounds within a structural class. Hence, rapid quantitation of compound libraries may be carried out with the use of a single external standard. Hsu et al. (*53*) reviewed the theory of ELSD and the design of commercial instruments. The application of ELSD to library analysis was illustrated using examples from the authors' library synthesis program. Complemented by UV detection for purity assessment and mass spectrometry for product identification, ELSD was the only technique affording sufficient accuracy and sensitivity for high-throughput library analysis. Fang el al. (*54*) examined 42 compounds from 7 different combinatorial libraries using a high-throughput LC/MS/UV/ELSD method and 33 commercial and standard compounds using both high-throughput and standard quantitation methods. It was demonstrated that compounds with low molecular weight (<300) are generally less responsive to ELSD, which can result in purity measured by ELSD appearing higher than by UV. Quantitation using a general UV calibration curve generally gives higher precision than using a general ELSD calibration curve. A calibration curve from structurally related compounds is needed for better quantitation. Fitch et al. (*55*) made one of the earliest descriptions on the use of LC/CLND for the assessment of solid-phase synthesis of combinatorial libraries containing nitrogen in the structure. Taylor et al. (*56*) evaluated a CLND detector as a universal quantitation tool for nitrogen-containing compounds. Using FIA and in conjunction with gradient HPLC, the CLND produced a linear response from 25 to 6400 pmol of nitrogen in the molecule for a set of chemically and structurally diverse compounds. In addition, the response was independent of mobile-phase composition. These results demonstrate that the CLND can be used with FIA or on-line with HPLC for rapid and accurate quantitation down to low-picomole levels, using a single external standard. The authors also demonstrated the combination of LC/UV/CLND/MS as a generic method for

rapid identification, quantitation, and purity assessment of small organic compounds. Shah et al. (*57*) developed a method for high-throughput quality control of combinatorial library compounds from parallel synthesis using a combination of FIA/MS and flow injection CLND. Compounds were characterized by mass spectrometry and concentration was determined by CLND with a throughput of 60 s/compound. Dulery et al. (*58*) developed a strategy of using a generic fast HPLC method with diode array detection (DAD) and MS to provide structure and purity information. In addition, complementary NMR analyses were done on selected compounds to provide a better structural characterization of the expected compounds and their potential side products. In a recent report, Yurek et al. (*59*) described the development and use of a new system for the simultaneous determination of identity, purity, and concentration of library components produced by parallel synthesis. The system makes use of HPLC with DAD, ELSD, CLND, and TOF-MS detectors (HPLC/DAD/ELSD/CLND/TOF-MS). The use of exact mass capability of TOF-MS along with CLND provides a synergistic combination enabling target and side-product structures to be identified and their concentrations and purities determined in a single analysis.

**Parallel LC/MS.** The MS instrumentation is the most expensive part of the LC/MS system; hence, efforts to improve the throughput of the LC/MS analysis often involve the use of parallel multiple columns feeding into a single mass spectrometer. Zeng et al. (*60*) developed an automated parallel analytical/preparative LC/MS workstation to increase the throughput for characterizing and purifying combinatorial libraries. The system incorporates two columns operated in parallel for both LC/MS analytical and preparative LC/MS purifications. A multiple sprayer ESI interface was designed to support flows from multiple columns. The system is under complete software control for delivering the crude samples to the two HPLC columns from a single autosampler. The authors demonstrated characterization of more than 200 compounds per instrument per day and purification of more than 200 compounds per instrument per night. De Biasi et al. (*61*) described a four-channel multiplexed electrospray LC interface coupled with an orthogonal TOF-MS capable of rapid data acquisition. The flow from a single HPLC pump was split to feed four columns simultaneously, and the flows from each of the four columns were coupled to the four-way multiplexed electrospray interface. The use of a high-resolution TOF-MS instrument provided unambiguous molecular weight assignment to both major components and synthetic byproducts. Wang et al. (*62*) described a similar parallel four-column four-way multiplexed electrospray interface. This setup enabled effluent flow streams from an array of HPLC columns to be sampled independently and sequentially into a single mass spectrometer. Effluent flow streams from an array of four HPLC columns are connected to a parallel arrangement of electrospray needles coaxial to the mass spectrometer entrance aperture. Sage et al. (*63*, *64*) reported a similar four-channel, as well as an eight-channel, source with the orthogonal acceleration TOF-MS system. A 96-well microtiter plate could be analyzed in 1 h by using a 5-min HPLC gradient with the eight-channel parallel LC/MS multiplexed electrospray interface system. Tolson et al. (*65*) reported a parallel LC system for a commercial multichannel electropray source. The performance of the Jasco PAR-1500 pumping system, which delivers an equal gradient flow to eight individual flow channels, was compared to that of a conventional system, where the flow is split postpump. Both systems were coupled to a LC/ESI-TOF-MS system equipped with a multichannel inlet system, used for the analysis of combinatorial libraries. In addition to flow stability, the Jasco system has resulted in a dramatic improvement in chromatographic performance.

Parallel LC/MS methods are also widely used in high-throughput quantitation for in vitro and in vivo pharmaceutical profiling of compounds. Bayliss et al. (*66*) described a parallel ultrahigh flow rate LC system using four columns in parallel and a four-way multiple sprayer interface to the mass spectrometer. This method was applied on both the narrow-bore and capillary-scale and enabled sensitive quantification of drugs from four plasma samples simultaneously, without sample preparation and with throughputs of up to 120 samples/h. Jemal et al. (*67*) investigated parallel-column liquid LC in conjunction with a conventional single-source ESI-MS. Within a single chromatographic run time, sample injections were made alternately onto each of two analytical columns in parallel at specified intervals. The sample throughput was increased by a factor of 2 compared with a conventional single-column approach. The dual-column and single-column methods were found to be equivalent in terms of accuracy and precision. Wu (*68*) developed a similar method of dual-column single mass spectrometric strategy and coupled the method with on-line extraction. This allowed for the direct injection of biofluids onto the system. The performance and capability of this system was shown to be comparable to those obtained on a conventional single-column system. Yang et al. (*69*) evaluated a four-channel multiplexed electrospray interface for the simultaneous validation of LC/MS/MS methods for the quantitation of the same compounds in four different biological matrixes. Performances in limit of quantitation, precision, accuracy, and intersprayer cross-talk for the multiplexed electrospray ion source were all satisfactory. Van Pelt et al. (*70*) developed a method incorporating four LC columns into a conventional system composed of one binary LC pumping system, one autosampler, and one mass spectrometer. Increased sample throughput was achieved by staggering injections onto the four columns, allowing the mass spectrometer to continuously analyze the chromatographic window of interest. Demonstrated precision and accuracy were well within the acceptance criteria. The parallel chromatography system decreased the overall run time from 4.5 to 1.65 min in comparison to a conventional LC/MS/MS analytical method. Recently, Xu et al. (*71*) reported an eight-channel parallel LC/MS system in combination with custom automated data processing applications for high-throughput early ADME study. The parallel LC/MS system was configured with one set of gradient LC pumps and an eight-channel multiple probe autosampler. The flow was split equivalently into eight streams before the multiple probe autosampler and recombined after the eight columns and just prior to the mass spectrometer ion source. The parallel LC/MS system is capable of analyzing up to 240 samples/h.

**Step Gradient LC/MS.** Further improvement of throughput in LC/MS analysis may be achieved by step gradient elution. This is in essence an on-line solid-phase extraction (SPE) process where the samples are loaded onto the column and washed with aqueous mobile phase, and compounds are eluted out with another

mobile phase of high organic content. The technique combines the simplicity of FIA with the benefit of removing impurities and buffer components before mass spectrometry detection. In this case, selectivity is achieved by mass spectrometry alone without chromatographic separation. The technique has been used for compound purity assessment and quantitation. An on-line back-flush SPE/MS technique has been used by Marshall for quality assessment of the combinatorial libraries (*72*). This back-flush elution procedure provides a very effective in-line removal method for the aqueous components in the matrix that are often responsible for ion suppression. Bu et al. (*73*) used a high-throughput analytical method for permeability screening of drug candidates. This simple and sensitive method was based on direct injection coupled with on-line guard cartridge extraction using a very steep gradient. The method relied on MS/MS to achieve selectivity without chromatographic separation. The authors used this method in a comparison study of apparent permeability across Caco-2 cells measured by sample pooling or cassette dosing strategy with results from single-drug dosing and discrete sample analysis. Janiszewski et al. (*74*) used a step gradient strategy in their dual-column LC/MS application on automated high-throughput quantitation. The strategy of collecting all data for a compound into a single file greatly reduced the number of data files collected, increased the speed of data collection, allowed rugged and complete review of all data, and provided facile data management. The described systems have analyzed over 40 000 samples/month for two years and have the capacity for over 2000 samples per instrument per day.

**Open Access Operation and Automation.** As FIA/MS and LC/MS become more pervasive in the analysis of combinatorial libraries, open access instrumentation is increasingly used in combinatorial laboratories as well as in support of general medicinal chemistry. These open access systems are most often used for reaction monitoring and optimization and, in some cases, for library quality control and synthesis product purification.

The first open access systems were described by Pullen et al. (*75*) and Tayler et al. (*76*). These systems were based on FIA-MS or generic LC/MS analysis with single quadrupole mass spectrometers to ensure the ease of use and ruggedness of the system. Spreen and Schaffter (*77*) also described an open access facility. The use of the open access facility has increased sample throughput by 60% and has allowed the mass spectrometrists to spent more time on nonroutine problems.

Automation of data acquisition, processing, interpretation, and reporting is another trend in the bid to improve the throughput of combinatorial library analysis. Greig (*78*) reported an automated procedure for calculating and storing the sample purity information based on the LC/MS results. The combination of a fast LC/MS method and automated data processing techniques enabled high-throughput analysis of combinatorial library samples at the rate of up to 2300 per week per instrument. Tong et al. (*79*) developed hardware components and software modules to enhance the automation, efficiency, and reliability of a commercial open access FIA/MS system. The software modules include utilities for data manipulation/reduction, data interpretation, data transmission, and reporting to the desktop computer of the submitter. Choi et al. (*80*) reported an approach to applying intelligent automation for high-throughput LC/MS analysis of compound libraries using Microsoft Visual Basic software. Compounds were analyzed by a generic primary HPLC method. Those that failed, in the initial analysis, were reanalyzed automatically using secondary analytical methods based on the information derived from the target analyte structure. Examples were described where a secondary method with a longer column and slower gradient was selected for target compounds that failed in primary analysis with ClogP values less than 1. Williams et al. (*81*) reported an automated molecular weight assignment method for positive ion ESI-MS spectra. The software application (MassAssign) differentiates $[M + H]^+$ ions from other signals in a complex mass spectrum and reports assignments in the forms of either a single component having the displayed molecular weight, multiple components, or a molecular weight undetermined. Initial testing of the program yielded a 90% success rate compared with manual interpretation for 55 samples.

**Analytical SFC/MS.** Supercritical fluid chromatography (SFC) is a technique similar to HPLC, varying primarily in that the mobile phase employed is a compressed gas with a gradient of organic modifier. Carbon dioxide is the most commonly used gas in SFC. Due to the lower viscosity of supercritical fluids, the chromatography can be run at much higher flow rates compared to HPLC. As a consequence, SFC can typically achieve higher chromatographic resolution or faster separation speeds than HPLC. SFC and HPLC can better be viewed as a continuum, both part of a larger unified chromatography (*82*). In practical application, a wide variety of different compositions of carbon dioxide and the organic modifier have been employed to achieve separation of components of interest. SFC has traditionally been applied to chiral separations owing to the increased resolution inherent in SFC relative to HPLC. Recently there is a growing trend in the application of SFC and SFC/MS to the analysis of combinatorial libraries.

Coupling mass spectrometry with SFC offers the potential benefit of high-speed separation and purification with sensitive detection and identification. In addition, the SFC mobile phase is considerably more volatile than the aqueous-based mobile phases that are typically used with reversed-phase LC/MS. This allows the entire effluent to be directed into the MS interface and simplifies the coupling of the SFC with atmospheric pressure ionization mass spectrometry including ESI and APCI.

One of the earliest reports of SFC interfaced with APCI was by Huang et al. (*83*). The authors used a pinhole restrictor to maintain supercritical fluid conditions in a packed column (pcSFC) system. Results for a mixture of five corticosteoids were described with an injection of 25 ng of each of the components. The system was also amenable for capillary SFC/MS applications with minimum modification. Sadoun et al. (*84*) reported an SFC interface with ESI in which a two-pump SFC and a packed column were used with the outlet directly interfaced to an ESI source of a quadrupole mass spectrometer. Also, 1−30% (v/v) of polar organic modifier (MeOH−$H_2O$ 95:5) was added to $CO_2$ mobile phase to help elute polar organic compounds. The setup was shown to allow analysis of polar organic compounds, which were difficult to analyze with earlier implementations of SFC/MS with a chemical ionization interface. A recent review article is available on pcSFC/MS (*85*).

Baker and Pinkston (*86*) modified an LC/ESI-MS interface for use with pcSFC. The use of a concentric sheath-flow liquid

provided ESI modifiers to assist the ionization of neutral, pcSFC-separated components. Postcolumn chromatographic fidelity was preserved by using a pressure-regulating fluid (supplied under pressure control) to the effluent just ahead of the sprayer. This modified interface has been used to characterize a variety of mixtures of compounds. Spectra produced by using the pcSFC/MS interface are similar to LC/ESI-MS spectra. Hoke et al. (*87*) compared a pcSFC/MS/MS method to an LC/MS/MS method for quantitation of enantiomers in human plasma. Samples were prepared using automated solid-phase extraction in the 96-well format. Generally, most analytical attributes, including specificity, linearity, sensitivity, accuracy, precision, and ruggedness, for both of these methods were comparable with the exception that the pcSFC separation provided a roughly 3-fold reduction in analysis time. A 2.3-min pcSFC separation and a 6.5-min LC separation provided equivalent, near-baseline-resolved peaks, demonstrating significant time savings for the analysis of large batch of samples using pcSFC. Hoke et al. (*88*) demonstrated the utility of pcSFC for high-throughput bioanalytical quantitation using dextromethorphan as a model compound. Plasma samples were prepared by automated liquid−liquid extraction in the 96-well format prior to pcSFC/MS/MS analysis. A throughput of ∼10 min/plate was achieved with acceptable RSD. Pinkston et al. (*89*) described a comparative study of 2266 diverse organic compounds using generic pcSFC ($CO_2$ with 5−60% MeOH gradient) and HPLC (3−95% ACN gradient) methods with ESI-MS detection and concluded that the range of coverage is comparable for the two techniques.

The Markides group at Uppsala University of Sweden was also among the earliest in the development of SFC/MS and demonstrated the application of the technique in the analysis of small organic molecules. Tyresors et al. (*90*) described an APCI-MS interface for an open tubular SFC system. The interface was designed to permit transport of the supercritical mobile phase into the ionization region of the mass spectrometer while maintaining its temperature to within 1 °C of the chromatographic oven temperature. Temperature control of the interface-transfer line was achieved using preheated gas streams from the chromatographic oven and an active electrical insulation. An average retention time reproducibility of 0.24% was demonstrated with an average 2.6% precision in relative peak height. Sjoberg and Markides (*91*) described a SFC interface probe for API-MS (including ESI and APCI). A sheath-liquid flow of 20 $\mu$L/min in ESI provides optimal conditions for both separation and ionization. The new probe also allows for an easy ionization mode change between ESI and APCI. Sjoberg and Markides (*92*) described improvement to the previous setup in order to obtain stable ion signals and better sensitivity. Factors that influence the ion signal intensity and stability have been studied including corona needle position, nebulizer gas flow and gas additives in APCI and spray capillary assembly dimension and position, liquid flow rate, and composition in ESI. The achievable detection limits were in the 50−0.1 pg (i.e., 290 fmol−140 amol) range. The detection limit in APCI mode was improved by a factor of about 20−25 compared to an earlier design (*91*).

Ventura et al. (*93*) have interfaced a SFC system to a mass spectrometer and evaluated the system for applications requiring high sample throughput. The authors demonstrated the high-speed separation and accurate quantitation capability of SFC/MS. The LC/MS analysis cycle time was reduced 3-fold using a general SFC/MS high-throughput method. Unknown mixture characterization was improved due to the increased selectivity of SFC/MS compared to LC/MS and was demonstrated with the analysis of combinatorial library mixtures utilizing negative ion APCI-MS analysis. Rapid elution of SFC was also shown to reduce both sample carryover and cycle time. In an extension of their early work (*94*), positive ion APCI-MS was used for the analysis of compounds with a wide range of polarities. Substituting SFC/MS for LC/MS results in substantial time savings, increased chromatographic efficiency, and more precise quantitation of sample mixtures. The instrumental setup also allows for facile conversion between LC/MS and SFC/MS modes of operation.

Morgan et al. (*95*) described a optimized interface for coupling SFC with APCI-MS. Data presented demonstrate that the internal diameter and length of the transfer line between the SFC unit and the APCI source are not critical in maintaining peak shape or retention time under the set of conditions tested. A comparison of responses from an in-line UV detector, two quadrupole mass spectrometers, and an ion trap was presented to demonstrate limits of detection and linear ranges for the SFC separation of a six-compound test mix. Villeneuve and Anderegg (*96*) developed an automated analytical SFC method to separate enantiomers based on a commercial instrument and column selection valves. Similar racemic compounds, even those from the same molecular class, were separated using different column and modifier combinations. Using the fully automated system, the optimal chiral separation of several compounds can be obtained unattended within 24 h. Garzotti et al. (*97*) described a simple and economical method to couple a commercial SFC system with a high-resolution hybrid mass spectrometer (quadrupole-TOF-MS). The setup provided on-line accurate mass SFC/MS measurements, and the fast spectral acquisition rate of TOF-MS facilitated data acquisition from rapid SFC separations.

**CE/MS.** Capillary electrophoresis (CE) is a powerful separation technique. It is especially useful for separation of ionic compounds and chiral mixtures. Mass spectrometry has been coupled with CE to provide a powerful platform for separation and detection of complex mixtures including combinatorial libraries. However, the full potential of CE in the application of routine analysis of samples including combinatorial libraries is still to be realized. This is in part due to perceived difficulty in the use of the CE technique compared to the more mature techniques of HPLC and even SFC. Dunayevskiy et al. (*98*) analyzed a library of 171 theoretically disubstituted xanthene derivatives using a CE/ESI-MS system. The method allowed the purity and makeup of the library to be determined: 160 of the expected compounds were found to be present, and 12 side products were also detected in the mixture. Due to the ability of capillary electrophoresis to separate analytes on the basis of charge, most of the xanthene derivatives could be resolved by simple CE/MS procedures even though 124 of the 171 theoretical compounds were isobaric with at least one other molecule in the mixture. Any remaining unresolved peaks were resolved by MS/MS experiments. The method shows promise for the analysis of small combinatorial libraries with fewer than 1000 components. Boutin et al. (*99*) used CE/MS along with NMR and MS/MS to characterize combinatorial peptide libraries containing three to four variable positions. The CE/MS method was used to provide a rapid and routine

method for initial assessment of the construction of the library. Simms et al. (*100*) developed a micellar electrokinetic chromatography method for analyzing combinatorial libraries with an open tube capillary and UV detection. The quick analysis time of the method made it suitable for the analysis of combinatorial library samples.

CE/MS was also used in the analysis of combinatorial libraries in affinity screening through the use of affinity capillary electrophoresis (ACE) (*101*−*104*).

## MASS SPECTROMETRY IN LIBRARY PURIFICATION

Libraries synthesized in a parallel format, whether they be on solid phase or in solution, generally require purification before being provided for biological assay. This necessity comes from the need to avoid any possible synergy or antagonism of multiple components in a crude reaction mixture, to avoid false positives or negatives due to assay interferences, and to provide reliable structure−activity relationships through the screening of confirmed structures. Mass spectrometry is employed as a primary validation tool in the purification of these libraries in one of two ways. Either the mass spectrometer may be employed on-line for analysis of each fraction as it elutes from the chromatogram or purified samples may be analyzed by either FIA/MS or by postpurification LC/MS to confirm identity. With HPLC, the most typical compound purification tool, a mass spectrometer, is thus used either as a detector for the compound of interest to initiate collection of peak with correct molecular weight or as a postcollection validation tool, via either FIA/MS or analytical LC/MS run. In the former case, the MS is integrated into the HPLC with a stream splitter and is continually on-line acquiring data. In the latter case, peak collection will be initiated (triggered) by another type of detector—most often UV or ELSD—and mass spectrometry will be enlisted to validate the desired component of the synthetic mixture.

**UV- and ELSD-Triggered Preparative HPLC.** UV-triggered collection was employed by Kibby (*105*) in the purification of combinatorial libraries by reversed-phase, normal-phase, or chiral chromatographic methods. This process entails an initial "scouting run" by analytical-scale HPLC, the conditions for which are selected by an analytical chemist based upon structural information supplied by the medicinal chemists. This initial analytical HPLC run is acquired with UV and APCI-MS detectors in order to verify the presence of desired reaction components, to determine whether purification is required, and to confirm that the selected analytical HPLC conditions are appropriate for subsequent purification scale-up. Further, this analytical "scouting run" also serves to select an appropriate collection window for subsequent UV-triggered preparative HPLC. The purification system is composed of Thermo Separation Products (TSP, San Jose, CA) HPLC pumps, autosampler, and variable-wavelength UV detector and an Isco (Lincoln, ND) fraction collector. The fraction collector, valves, and analog-to-digital converter are controlled by custom software, and the TSP software coordinates the pump, autosampler, and detector actions. The sample ID, UV, and MS data as well as chromatographic run-time data are all imported from the analytical HPLC system into the purification software package. Only when UV threshold parameters are met within the appropriate selected collection window are peaks collected, thus limiting the number of fractions per sample. A log file for fractions collected is generated by the combinatorial purification software, providing a record of peaks and their corresponding rack and tube locations. The desired component, and the corresponding fraction collection tubes, are identified from these data by a comparison of the preparative chromatogram to the prepurification analytical run for each sample. If required, purified samples are subsequently submitted to FIA/MS analysis. Multiple column sizes allow the system to accommodate the purification of samples sized up to 50 mg in weight.

Searle et al. employed a UV-triggered or ELSD-triggered preparative HPLC system (*106*, *107*) for purification on a service basis of samples provided by both medicinal and combinatorial chemists. Samples are first triaged for purification on a series of open access analytical LC/MS instruments with standard methods. Once the synthetic chemist has ascertained that reaction mixtures both contain the desired component and require purification, these analytical data, along with samples, are submitted to the purification group. The purification chemist then selects one of several standard preparative HPLC methods based upon the analytical HPLC retention time. Either UV- or ELSD-triggered collection is used based upon the reliability of the triggering methods for the desired component. The system is composed of Waters (Milford, MA) HPLC pumps, autosampler gradient makers, DAD, Gilson (Middleton, WI) fraction collectors, and an Alltech (Deerfield, IL) ELSD detector. The preparative HPLC system can be used to accommodate the purification of samples ranged from 10 to 300 mg in weight by selection of appropriate UV detector flow cell path lengths, column sizes, and prefraction collector delay loops, with two options of each. In this setup, four individual preparative HPLC instruments are fed into a single MS for validation by either ESI or APCI and either positive or negative mode, dependent upon the structure of the desired component. The motivation for this format lies in the fact that a single faster, yet more expensive instrument—the mass spectrometer—can be employed to process samples easily from four low-cost, yet slower process instruments—the HPLCs. Customized software developed in-house provides sample tracking capabilities, which begins with an intranet-based submission system allowing the synthetic chemist to provide sample ID, sample weight, and molecular weight of the desired component(s). Purification chromatograms are imported onto the purification chemist's desktop into a data handling package from which fractions may be selected for MS based upon a comparison of the analytical and preparative HPLC chromatograms. These samples are then placed on a Gilson liquid handler front end to a Finnigan (San Jose, CA) LCQ-MS and the appropriate tube and rack location selected by the software for MS analysis. An aliquot from each sample is delivered to and analyzed by FIA/MS, the spectra from which are automatically exported back into the postpurification analysis software, where final sample selections are made and the corresponding fractions dried, transferred to weighed vials, and returned to the synthetic chemists along with a purification report.

Weller et al. (*38*, *108*) first developed an open access automated preparative HPLC system based upon UV-threshold triggered fraction collection. This system was composed of Shimadzu (Columbia, MD) pumps and system controller, variable-

wavelength UV detector, a modified Shimadzu autosampler, and series of Shimadzu fraction collectors. By employing fast flow rates and short columns, this system can purify up to 200 samples/day up to 200 mg in weight and operates in an unattended mode, with gradient cycle times as low as 7 min/sample. Customized software and hardware were developed including an algorithm that optimizes system time by analyzing gradients employed in subsequent samples to determine whether a sample can be applied via an "inject ahead" method, reducing the injection overhead from 90 to 5 s between samples.

Schultz et al. (*109*) and Coffee (*110*) codeveloped a "walk-up" parallel-format UV-triggered fraction collection purification system which they employ for the purification of their parallel solution-phase libraries designed for maximum diversity. Libraries accommodated by this system are all synthesized on the 1 mM scale and provided to purification in a 48-well microtiter plate format. High-efficiency purification is accomplished through UV-triggered fraction collection by employing four HPLC columns and four injection systems run in parallel with identical gradients. These are accommodated by a single, custom-designed UV detector with four flow cell paths. Any two wavelengths can be used simultaneously to trigger collection by either threshold or slope parameters. Samples thus generated are each subjected to FIA/ELSD/MS analysis to confirm identity and estimate recovery, respectively. ESI mass spectra are analyzed in both positive and negative ion modes on a PE Sciex (Concord, ON, Canada) instrument and ELSDs on a Sedere (Alfortville, France) via a Gilson liquid handling system simultaneously from a split stream. Custom-developed software tracks samples throughout a highly integrated process. The desired components are then clustered to a new microtiter format for biological analysis. Interestingly, components other than the desired reaction product in each case are also retained for "alternative screening".

**MS-Triggered Preparative HPLC.** An advantage of employing molecular weight rather than UV or ELSD response to trigger fraction collection from a crude reaction mixture lies in the fact that only a single component, most often all that is desired from most parallel library syntheses, is collected. This method reduces the fraction collection capacity required for the purification of large libraries and simultaneously provides validation of structure on-line during the compound purification, thus requiring no postpurification analysis. An excellent discussion as to the relative merits of UV versus MS detection to trigger fraction collection in various environments is provided by Kassel (*9*).

Zeng et al. (*42*) developed the first MS-triggered HPLC system for the purification of their combinatorial chemistry libraries. The authors set up a dual analytical−preparative HPLC system with parallel configuration of columns (which they termed "parallel Analyt/PrepLC/MS"), through hardware modifications to commercial instrumentation. This system has the capacity to analyze and purify more than 100 samples/day. Initial analytical LC/MS chromatograms acquired on this system allow identification of those samples requiring purification. Reaction mixtures are subjected to LC/MS with UV detection incorporated, and the percentage of desired compound is estimated by a postprocessing script which averages the intensity of UV peaks at both 220 and 254 nm. Those judged by this technique to be <85−90% pure are subsequently subjected to preparative HPLC on the same instrument. The preparative HPLC system is composed of Shimazdu HPLC pumps (used for both analytical and preparative systems) and autosampler, a Gilson fraction collector, and a PE Sciex (Thornhill, ON, Canada) mass spectrometer with ESI source as the only detector on-line during purification. Fraction collection is triggered only when an ion of desired $m/z$ is observed above a preset threshold level. Therefore, only the compounds of interest are collected, and validation of identity is accomplished simultaneously via the on-line mass spectrum. This technology is available to synthetic chemists on an open access basis, where the user may select upon walk-up either the analytical or the preparative HPLC mode. Short columns and high relative flow rates, along with varying column sizes, flow rates, and fraction collector tubes, are employed to achieve the rapid separation on a preparative or semipreparative scale of samples varying from 1 to 100 mg in weight. Collection parameters are set by the user, and purification yields range from 50 to >90% based upon chromatographic behavior of individual compound types. A later modification to this system allowed an improvement in throughput to the initial dual analytical/preparative column system (*60*, *111*). The modified parallel system varies from the system first developed by the group in the incorporation of each of two of the analytical and preparative HPLC columns, thus expanding the instrument capability to accommodate the analysis of >200 compounds/day and the purification of >200 compounds/night. The HPLC pump flow is split equally between two identical preparative columns, and the automated switching valves incorporated allow application of samples from separate microtiter plates to each of the preparative columns. Similarly, modifications to the mass spectrometer allow the single MS to monitor the eluant from two columns simultaneously (analytical as well as preparative). A minor limitation to this system lies in the necessity that the desired compound from parallel libraries to be purified must have unique masses in samples applied from corresponding wells of the two microtiter plates which are purified simultaneously. This seems a minor tradeoff for the doubled throughput per instrument. In cases where only a single product of desired molecular weight is collected from each member of a parallel library, no sample tracking is required, as injection and collection footprints will be identical, with four microtiterplates generating 384 fractions, or four microtiter plates or tubes of corresponding compounds.

An integrated analytical- and preparative-scale LC/MS system was employed for the purification of solution-phase parallel libraries by Diggelmann (*112*, *113*) in support of agrochemical research. The purification is accomplished by mass-triggered HPLC on a system wherein analytical and preparative HPLC systems are setup in parallel, either of which can be sent through MS, UV, and ELSD detectors. After analytical LC/MS runs for each crude parallel synthesis mixture, a decision algorithm is employed to triage samples for purification, doing ELSD-based quantitation, and categorizing each sample into pure desired products, pure unknowns, mixtures with desired component, and mixtures composed only of unknowns. This analytical work is then followed, on the same dual system, by mass-triggered HPLC purification with the requisite delays and makeup flow similar to that developed by Weller (*38*). This system is capable of purifying up to 200 samples/day. On an interesting note, the authors also collect and retain samples for screening from the purification

efforts other than those corresponding to the desired product in order to maximize synthetic productivity efforts.

Kiplinger et al. (*114*) designed a system to accomplish the purification of samples in the 10−20 mg level that could be employed in either the MS-triggered or UV-triggered format, or both. Custom-packed Phenomenex (Torrance, CA) short columns (10 mm × 50 mm) with alternating column regeneration cycles allow the purification of a 96-sample set in a 16-h overnight run. The system, is composed of Hewlet-Packard (Palo Alto, CA) HPLC pumps and photodiode array detector, an Alltech ELSD, a Gilson fraction collector and liquid handlers, and a Micromass (Manchester, UK) mass spectrometer. The mass spectrometer control program provides the peak trigger signal based upon output from either a diode array UV detector or the mass spectrometer, and an additional "slaved" program controls the fraction collector. A series of stream splitters and a makeup pump for flow to the mass spectrometer are employed in order to deliver appropriate concentrations of sample into the mass spectrometer, which can be operated in either APCI or ESI mode. Waters offers a commercially available instrument at this time based upon this design.

Thomas et al. (*115*) developed a MS-triggered semipreparative (typically 10 $\mu$M of sample to be purified) HPLC purification system in support of their parallel library syntheses. This system is composed of Shimadzu HPLC pumps and UV detector, Gilson liquid handlers, and a PE Sciex ESI-MS. This system accommodates a 96-well plate of samples in a 24-h period. Initial characterization and postpurification analysis LC/MS runs are performed on an eight-channel high-throughput system, able to process a 96-well plate in 90 min.

**Preparative SFC/MS.** Supercritical fluid chromatography, as has been discussed in the analytical section of this review, is a technique similar to HPLC, varying primarily in that the mobile phase employed is compressed carbon dioxide gas with a gradient of organic modifier. The implication of this mobile phase to preparative-scale chromatography lies in the fact that, upon experiencing a pressure drop postcolumn and just prior to fraction collection, the carbon dioxide reverts to the gas phase. This delivers purified sample dissolved in only minimal amounts of organic cosolvent, generally methanol, which can be more rapidly and more gently removed than the typical large volumes of aqueous organic solvent provided by reversed-phase HPLC purification formats. Preparative-scale SFC is only now beginning to realize its potential in the purification of combinatorial chemistry libraries, but much as with HPLC, mass spectrometry seems poised be an integral component of the separation and validation processes.

Berger Instruments (*116*), working with Alanex (now Pfizer Global R&D/Agouron Pharmaceuticals) scientists (*117*), developed the first preparative-scale SFC instrument to be employed for the purification of combinatorial chemistry libraries. The preparative SFC system which Berger Instruments (Newark, DE) provides is composed of several modified or developed components. Varian (Walnut Creek, CA) HPLC pumps, one of which is highly modified to achieve accurate delivery of carbon dioxide, a compressible fluid, and a high-pressure mixing column are used to deliver mobile phase. Injection is achieved manually with a Valco valve onto a normal-phase preparative or semipreparative column where sample detection in the Berger system is achieved by a Varian UV detector modified for high-pressure flow. The column outlet pressure is controlled via back pressure regulation downstream of the UV detector, and a Berger Instruments "separator" prevents aerosol formation as fluid-phase methanol/carbon dioxide expands to the gas phase and separates from the liquid organic modifier. Fractions are collected at elevated pressure into a "cassette" system composed of four individual compartments, each with a glass collection tube insert, allowing efficient collection of up to four components per chromatogram.

Farrell et al. (*117*) described a process that uses an analytical SFC/MS/CLND and a preparative system consisting of a CLND off-line of the preparative SFC/MS for the purification of their parallel solution-phase library syntheses for both lead discovery and optimization. The off-line system is composed of the Berger Instruments preparative-scale SFC system, an Agilent (Palo Alto, CA) LC/MS (LC/MSD), and a Sievers (Boulder, CO) CLND. An auxiliary pumping system is included in order to provide a constant flow to the CLND, which is required to achieve good quantitation data on-line. Prepurification analytical SFC/MS/CLND allows the triage of samples for purification, and an in-house software package analyzes data for predicted quality based upon an evaluation of UV and MS data for the potential of coeluting peaks during purification. This same software package selects a collection time window for purification, which is necessary to limit the number of fractions per sample. This system accommodates the purification of samples up to 50 mg in weight. Postpurification analytical SFC/MS/CLND is used as well to validate purified samples.

Pan et al. (*118*, *119*) modified a Berger Instruments preparative SCF for use in support of the purification of libraries synthesized by a high-throughput organic synthesis group and have integrated this system into the existing preparative HPLC purification processes. A "manual" version of the Berger instrument was integrated with a Gilson autosampler and a Cavero (Sunnyvale, CA) pipetting instrument customized in-house to serve as a fraction collector. A custom-designed fluid/gas outlet or "shoe" on the fraction collector enables collection of samples at atmospheric pressure. A methanol wash system is incorporated into the fraction collection line to ensure high recovery and eliminate cross-contamination between fractions. Fraction collection is UV-triggered and postpurification validation is accomplished by mass spectrometry on a Finnigan LCQ-MS fitted with an APCI probe in the positive or negative ion mode as is most appropriate to the structural class being isolated. An in-house software package, written for the UV-triggered, MS-validated preparative HPLC setup already in place, was expanded to accommodate SFC data. This software tracks mass spectrometry results and fraction collection data, correlating test tube location within a rack to chromatogram peaks as well as moelcular weight. Thus, samples purified on each of four HPLCs and one SFC by UV trigger, are each validated on a single MS utilizing identical footprint fraction collector racks and via a single software package.

Wang et al. reported (*120*) the development of a preparative-scale SFC with mass-directed fraction collection capabilities. This system is composed of a Gilson preparative SFC and a Gilson autosampler, which is used for both injection and collection, and has been modified by replacing the standard valves with high-

pressure-rated valves. MS detection is accomplished with a PE Sciex (Foster City, CA) ESI-MS which is operated in the positive ion mode. To improve recovery into the fraction collection system, a simple foil seal is placed over the collection tubes, providing a loose seal to encourage retention of the methanol portion of the eluant and departure of the carbon dioxide. Two solvent makeup pumps were added to the system. One pump was added to provide additional flow to the fraction collector as carbon dioxide departs and a second to provide auxiliary flow of methanol containing formic acid additive to the mass spectrometer stream in order to dilute sample concentration as well as to improve ion signal and peak shape. Fraction collection is triggered when the ion signal for a component of the desired molecular weight from each sample goes above a preset threshold value and is controlled through the mass spectrometer software package. This system is capable of purifying crude samples up to 50 mg in weight for chromatographically well behaved samples and used for chiral separations as well as general library purifications. Kassel has also reported (*121*) preliminary efforts toward a MS-triggered preparative SFC system on the Berger platform.

Ripka et al. (*122*, *123*) have custom designed a mass-triggered preparative-scale SFC system for purification of their parallel synthesis libraries. This is a parallel four-channel system capable of processing four microtiter format plates simultaneously. A unique protocol is used wherein UV detection is first employed to identify peaks from each sample as they elute, followed by diversion of a split stream from the eluant to a mass spectrometer for molecular weight determination. Each of the four SFC columns is monitored by a dedicated UV detector, but as peaks are identified by their respective detectors, all are diverted to a single TOF-MS. Fraction collection is initiated by the mass spectrometer, which identifies the desired target peak, and then triggers fraction collection into a "target plate". In addition to collection of the desired component, reaction byproducts are also diverted into an ancillary plate whose fraction collection is initiated through identification by the mass spectrometer of a molecular weight other than that of the desired product. Collection plates are a 2-mL deep well microtiter format and are custom designed with "expansion chambers" to accommodate evaporation of carbon dioxide as it departs. A 31-s "timeout" at the flow rate of 12 mL/min is used in order to avoid collection above a desired volume per sample and to achieve collection of samples into single wells. In-house-designed software tracks all compounds collected with respect to plate and well location.

## CONCLUSIONS AND NEW DEVELOPMENTS

Combinatorial chemistry has become an integral component of nearly all drug discovery efforts alongside other new technologies such as high-throughput screening and genomics/proteomics. Mass spectrometry coupled with separation techniques such as HPLC has become an important enabling technology for combinatorial library analysis in drug discovery. Parallel processing, open access and automation in the analysis, data processing, and interpretation and reporting of results have been the major trends in the recent years in the analysis and purification of combinatorial libraries. This trend should continue to strengthen for the foreseeable future. The substitution of an SFC front end to MS in lieu of HPLC has been a growing trend in combinatorial library analysis. It is possible that the use of SFC/MS will be extended to the evaluation of in vitro and in vivo pharmaceutical profiling of library compounds such as ADME/tox, CE/MS has not been used widely in the analysis of combinatorial libraries. To date, the application of CE/MS was mostly in the analysis of mixture component libraries derived from split synthesis and in the affinity screening of libraries through ACE. However, with the miniaturization of biological screening in the "lab-on-a-chip" format, CE/MS may find renewed interest due to the ease of coupling CE with miniaturized biochip separation platforms (*124*−*127*). Thus, miniaturization and chip-based technology may be an important new development for the analysis of combinatorial libraries in conjunction with the biological testing of these compounds. Trends in library purification seem to be directed more toward mass-triggered collection technologies in place of UV-, ELSD-, or CLND-triggered collection. Further, SFC has seen a growing acceptance in preparative purification of combinatorial libraries and has already been coupled to provide a MS triggered format.


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## ABBREVIATIONS

| | |
|---|---|
| ACE | affinity capillary electrophoresis |
| ACN | acetonitrile |
| ADME/tox | absorption, distribution, metabolism, excretion, and toxicity |
| API | atmospheric pressure ionization |
| APCI | atmospheric pressure chemical ionization |
| CE | capillary electrophoresis |
| CLND | chemiluminescent nitrogen detector |
| DAD | diode array detector |
| ELSD | evaporative light scattering detector |
| ESI | electrospray ionization |
| FIA | flow injection analysis |
| HPLC | high-performance liquid chromatography |
| LC | liquid chromatography (synonymous with HPLC in this review) |
| MS | mass spectrometry |
| MS/MS | tandem mass spectrometry |
| $m/z$ | mass-to-charge ratio |
| NMR | nuclear magnetic resonance |
| pcSFC | packed column supercritical fluid chromatography |
| SFC | supercritical fluid chromatography |
| SPE | solid-phase extraction |
| TOF-MS | time-of-flight mass spectrometer |
| UV | ultraviolet |

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