Combinatorial Chemistry and Mass Spectrometry in the 21st Century Drug Discovery Laboratory

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I. Introduction

Combinatorial chemistry is widely viewed by the pharmaceutical, agrochemical, and biotechnology industries as a key technology for accelerating the discovery of novel therapeutic agents. The term combinatorial chemistry has taken on many different meanings, but in a general sense, it represents a new wave of "parallel" thinking by both medicinal and synthetic chemists as a means of accelerating their discovery efforts. Combinatorial chemistry has its origins dating back to the seminal work of Merrifield,[1] where solid-phase synthesis protocols were used to generate small mixtures of peptides on solid support. This early work laid the foundation for the field of combinatorial chemistry and peptide libraries. Pioneers in the field of combinatorial chemistry,[2] such as Houghten and Geysen, capitalized on the wealth of information available by Merrifield and others on solid-phase peptide synthesis and applied it in a very unique way, that is, to perform peptide coupling reactions in parallel. These peptide libraries were

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Dr. Kassel received his Ph.D. degree in Analytical Chemistry at Michigan State University in 1988. Directly following, he held a postdoctoral fellowship at The Massachusetts Institute of Technology studying under the tutelage of Professor Klaus Biemann. In addition, Dr. Kassel was a recipient of a two-year NIH National Research Service Award fellowship in the Departments of Molecular Biology and Human Genetics at Harvard University. Following his postdoctoral studies, Dr. Kassel joined Glaxo Wellcome, Inc., where he held, over the course of his tenure, steadily increasing roles of responsibility within the Departments of Bioanalytical & Structural Chemistry. During this time, he pioneered the coupling of perfusion chromatography and mass spectrometry for the rapid characterization of proteins and enzyme digests as well as elucidating the role of phosphorylation in protein signaling pathways. Dr. Kassel also pioneered affinity LC/MS for assessing protein:ligand interactions. In November of 1995, Dr. Kassel joined CombiChem, Inc. as Director of Analytical Technologies. His research team developed state-of-the-art analytical tools to aid the drug discovery process. His seminal contributions to the field of combinatorial chemistry have included the design, development, and implementation of automated preparative HPLC/MS for the purification of compound libraries. In addition, his team developed an array of parallel HPLC/MS systems for ultrahigh-throughput analysis, purification, and in vitro ADME profiling of combinatorial libraries. Dr. Kassel has co-authored approximately 40 manuscripts, principally in the area of LC/MS, served as a reviewer for numerous journals, and is an Editorial Board member of *Combinatorial Chemistry & High Throughput Screening*. He has been an invited speaker at numerous conferences both nationally and internationally, has chaired several mass spectrometry conferences, and has organized several short courses and workshops, including the well-received ASMS short courses on "Mass Spectrometry of Peptides and Proteins" and "Mass Spectrometry and Combinatorial Chemistry". In November of 1999, CombiChem, Inc. was acquired by Dupont Pharmaceuticals, maintaining their west-coast operations in sunny San Diego. Dr. Kassel and his team continue to develop state-of-the-art technology for drug discovery. Recent efforts have focussed on implementing in vitro ADME profiling very early into the discovery process to reduce the likelihood of compound attrition as compounds advance toward nomination status.

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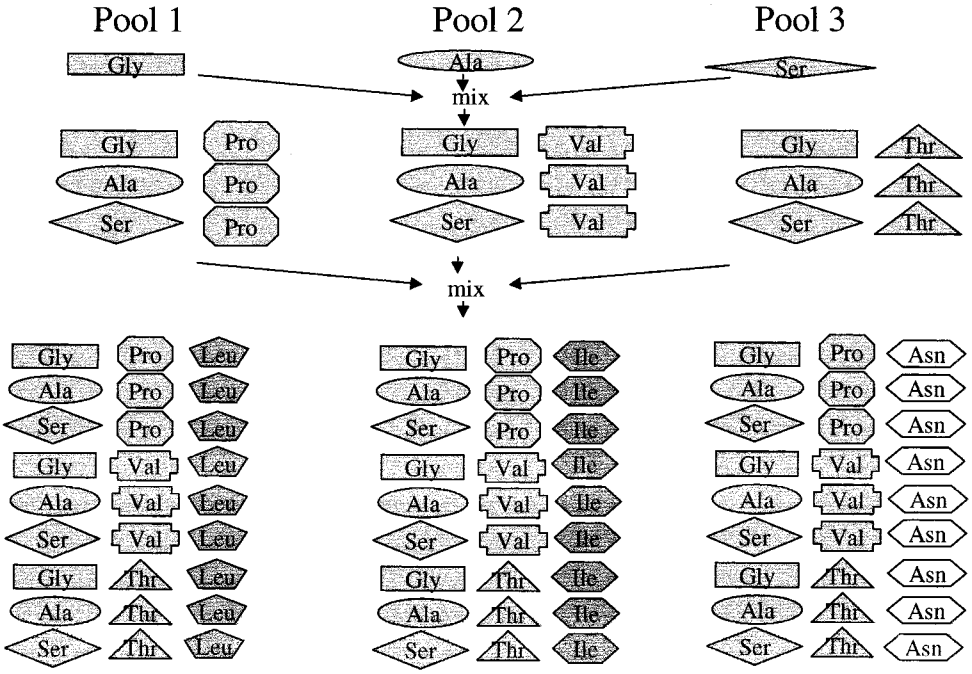


Figure 1. Schematic representation of the split and mix technique to produce combinatorial libraries.

prepared as mixtures using, what is referred to as, split−couple−recombine (or split and mix) methodologies. The process of split and pool synthesis is pictorially represented in Figure 1. In this example, activated polymeric resin (e.g., Rink resin) is distributed evenly into three separate reactors (i.e., pools). Pool 1 is exposed to a large excess of a single, unique amino acid (e.g., glycine) such that glycine is successfully coupled to the individual polymeric beads contained within this pool. Pool 2 is treated with a large excess of another unique amino acid (e.g., alanine) such that alanine is successfully coupled to the individual polymeric beads contained within pool 2. Similarly, yet another unique amino acid (e.g., serine) is coupled to the beads contained within pool 3. Next, the "pools" are recombined and after this divided evenly into three distinct pools again. The process is repeated, this time using a new set of three monomers (in this example, the amino acids proline (Pro), valine (Val), and threonine (Thr). The process is repeated a third time using another set of unique amino acids (in this example, leucine (Leu), isoleucine (Ile), and asparagine (Asn), resulting in a total of 3^3 (or 27) unique tripeptides. The true power of combinatorial chemistry lies in the ability to generate exquisitely large collections of peptides incorporating an identical process. For example, a pentapeptide library, starting with an activated resin and where all 20 natural amino acids are added sequentially at each step of the split−couple−recombine process, leads to a library of 20^5 (or 3 200 000) compounds! The work of Geysen et al. and Houghten et al. elegantly demonstrated the power of combinatorial peptide library synthesis and the utility of these very large peptide libraries in defining antigenic determinants and minimal protein binding epitopes[3,4]

However, early experiences with the split and mix combinatorial chemistry approach in the drug discovery setting were disappointing. As will be described later, this is due principally to an inability of reliably and routinely determining the active component(s) responsible for the biological or pharmacological activity from the mixture without significant effort and cost. Around 1994, groups began to abandon the split and mix combinatorial chemistry approach in favor of high-speed, spatially addressable automated parallel solid-phase and solution-phase synthesis of discretes.[5−7] Both solution-phase and solid-phase parallel synthesis permitted the production of large numbers of discrete compounds as well as large quantities of these discrete compounds, eliminating the need for extensive decoding of mixtures and resynthesis following identification of "active" compounds in high-throughput screening.

Importantly, parallel synthesis could be performed readily in microtiter plate format amenable to direct biological screening. Several groups developed proprietary reactor blocks, some amenable to full automation[8−10] and some for use by the bench chemist as a means for significantly increasing compound output relative to traditional medicinal chemistry capabilities. The relative ease of automation of parallel synthesis led to a tremendous in flux of compounds for lead discovery and lead optimization. Whereas high-throughput bioassay development was maturing at the onset of high-throughput parallel synthesis and combinatorial chemistry, analytical tools for combinatorial library characterization lagged visibly behind.

Almost all of the analytical characterization tools (e.g., HPLC, NMR, FTIR, and LC/MS) are serial-based techniques, and parallel synthesis is inherently parallel. Consequently, this has led rapidly to a new bottleneck in the discovery process (i.e., the analysis and purification of compound libraries). Parallel synthesis suffers from some of the same shortcomings of split and mix synthesis (e.g., the expected compound may not be pure, or even synthesized in sufficient quantities). The analytical community was faced with the decision of how to analyze these

parallel synthesis libraries. This review focuses on the analytical challenges associated with the field of combinatorial chemistry. In particular, this review highlights mass spectrometry and the many ways it is being applied to the very diverse field of combinatorial chemistry.

II. Analysis and Deconvolution of Combinatorial Libraries

A. Analysis of Peptide Libraries by Mass Spectrometry

Although peptide libraries are relatively easy to prepare, determining their composition is less readily accomplished.[11] Mass spectrometry, incorporating either electrospray or APCI ionization, has been used to qualitatively assess the fidelity of peptide library synthesis. Knowledge of the monomers (i.e., amino acids or synthons) used in constructing the peptide libraries allows for a theoretical library whose composition can be determined readily. An example of the experimentally measured electrospray spectrum of the synthesized peptide library is shown in Figure 2A. The theoretical (or simulated) electrospray spectrum of a 400-component peptide library is shown in Figure 2B. On the basis of the qualitative similarities between the theoretically and experimentally derived mass spectra, it may be concluded that the split and mix combinatorial peptide library synthesis was performed successfully and is amenable to direct biological screening. In some, cases, however, significant differences between the experimentally derived and theoretical mass spectrum are observed. Most frequently, the differences can be attributed to under (or over) coupling of monomer/reagent at various stages of the synthesis.

Peptide libraries were used to identify substrates for serine, threonine, and tyrosine kinases and phosphatases as well as for identifying peptide specificity for binding to SH2 domains of tyrosine kinase signaling proteins, such as Src tyrosine kinase (Src) and epidermal growth factor receptor kinase (EGFR). In these studies, probe libraries, synthesized with tyrosine and phosphotyrosine, were used to determine optimal Src and EGFR substrate sequences. The molecular weight envelopes for the unphosphorylated and phosphorylated peptide libraries should (theoretically) differ by 80 Da. These differences were clearly measured by electrospray ionization mass spectrometry. In these studies, gas-phase Edman sequencing and electrospray ionization mass spectrometry were used to identify the optimal (or preferred) substrates.[12−14]

Chemical libraries, unlike their peptide library predecessors, relied heavily upon mass spectrometry as an analytical characterization tool.[15] Whereas peptide synthesis on solid support and the coupling efficiencies (i.e., reactivities) of natural amino acids were well studied and understood, coupling efficiencies for nonnatural amino acids, organic acids, and amines were far less understood. These early organic compound libraries were found to contain far fewer desired products than previously observed with peptide libraries, and the relative ratios of the synthesized products varied dramatically. Differences in the coupling efficiency of various side chains caused these libraries to be problematic for traditional decoding strategies. Consequently, mass spectrometry became the tool of choice for characterizing synthetic combinatorial libraries. Numerous examples of combinatorial libraries characterized by a variety of mass spectrometry techniques, including quadrupole mass spectrometric analysis, high-resolution FT-MS, and matrix-assisted laser desorption ionization (MALDI) have been reported.[16−19] Almost invariably, these mass spectrometric analyses revealed that the composition and purity of the designed library was not achieved, the most frequently observed problem being that of partial and/or incomplete coupling in the split and mix approaches.

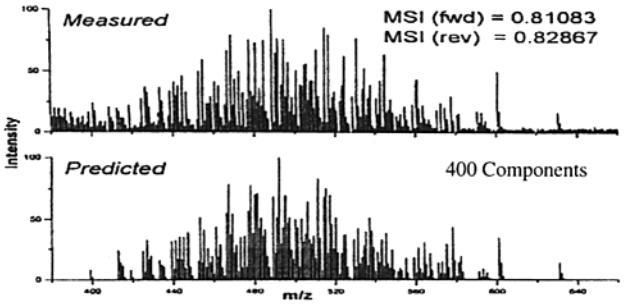


Figure 2. (A) Experimentally measured mass spectrum of a 400-component library. (B) Predicted mass spectrum of the same library synthesized by split−couple−recombine methodology. A statistical comparison of the two spectra was made, showing a 0.81−0.82 correlation (a correlation value of 1.00 corresponds to an exact match between predicted and measured spectra). (Reprinted with permission from Yates, N. et al. Proceedings of the 46th ASMS Conference on Mass Spectroscopy and Allied Topics, Orlando, FL, June, 1998; p 1042.)

B. MS Analysis of One Bead−One Compound Libraries

The inability to prepare combinatorial organic library mixtures in quantitative yields and the significant time required to decode the active compounds[20] led to the "one bead, one compound" approach. The split and mix methods are a facile way of generating very large libraries, where only one compound is synthesized per bead. Great effort has been placed on robotic sampling, bead picking technology, and the spatial array of the individual beads in microtiter plate format for biological testing. In the one bead−one compound approach, active wells were decoded subsequently by mass spectrometry strategies.[21,22] Sepetov and co-workers showed that for a 60 000-member spilt−couple−recombine library, in which individual beads were screened for biological activity, mass spectrometry could be used to identify the mass of the compound of the active bead.[23] Unfortunately, because split and mix approaches inherently lead to tremendous molecular weight degeneracy,[24] the mass of the compound on the active bead identified could eliminate only a portion of the library from further consideration. MS/MS, on the other hand, provided the selectivity (and sensitivity) to permit full decoding and identification of the active species in their particular example. Similarly, Brum-

mel and co-workers illustrated compound library deconvolution by MALDI and post-source decay-MALDI and concluded that tandem mass spectrometry "virtually eliminates the need for other decoding strategies...".[22]

C. Mass Spectroscopy Based Encoding Strategies

For non-peptide combinatorial libraries, MS/MS information alone is often insufficient to provide absolute confirmation and identity of compound on the bead. Recently, the tandem mass spectrometry based strategies have been complemented by elegant coding strategies set forth by several groups. Attaching a "tag" or "code" to the individual molecule associated with a single bead has led to an array of new analytical tools for their characterization that are neither as labor intensive or costly. One approach, described by Connely et al.,[25] involves attachment and subsequent release of a molecular tag that is capable of being identified by gas chromatography. Fitch et al.[26] incorporated an acid-labile linker and demonstrated that these tags could be decoded with high speed and sensitivity by LC/MS, reducing the analysis time per active bead to <2 min/sample. Geysen et al.[27] developed a mass spectrometry based decoding strategy incorporating isotopically labeled tags (^{13}C and ^{2}H) onto the beads containing the desired product.

Each of these tagging methods permits much more rapid decoding of combinatorial libraries than the recursive deconvolution approach described by Houghten et al.[3] However, the coding methods suffer from some major limitations and shortcomings. First, each of the methods is an indirect decoding method. (i.e., each elegantly identifies the "expected" product on the bead but does not confirm its presence and/or purity). Thus, subsequent resynthesis and bioassay testing is required. Second and perhaps a less significant problem is that the code itself can interfere with or contribute to false positives/negatives in the bioassay (each code, being a unique organic molecule, has a potential binding influence). To circumvent this problem, some groups run assays in parallel, whereby the code is partially released/cleaved before the assay. An additional liability to the single-bead strategy is that in the absence of very elegant robotic systems for bead transfer and very integrated data management systems, this process is logistically challenging. Finally, single-compound per bead strategies allow for only very small quantities of compound to be synthesized (e.g., a 100 μm bead typically is capable of generating approximately five nmol of pure product). Although these quantities are typically more than sufficient to perform myriad biological assays (especially with the recent introduction of 384-well and 1536-well assay plates[28,29]), they are not typically sufficient to permit extensive analytical characterization and structural elucidation (i.e., ^{13}C NMR, 2D-NMR, and tandem mass spectrometric analysis). Furthermore, they may present challenges in the purification, fractionation, evaporation, and quantification (reweighing) process. Irori has developed a radio frequency tag technique to overcome the issue of small quantity of sample per bead, enabling multimilligram quantities of individual components to be synthesized in MicroKans.[30] Frequently, the tag encodes for the expected product but the expected compound on the bead is either not what it is believed to be, not in a pure form, or not present at all. In any of these instances, a post-screening analytical assessment of the expected product is required.

D. Direct Bead-Bound Analysis Mass Spectrometry

Bead-bound materials are capable of being analyzed directly by techniques such as secondary-ion mass spectrometry,[31,32] MALDI-TOF-MS,[33] and FT-IR and magic angle spin NMR,[34] where the latter two techniques were used in combination to assess estrogen receptor library formation. Importantly, direct bead-bound analysis affords an effective means for monitoring of the progress of combinatorial chemistry reactions. More typically, the samples are attached to the bead via a photolabile or acid-labile linker and a fraction of the desired product is liberated during the course of (or at the end of) the reaction and characterized by flow injection (open access) mass spectrometric analysis.

E. Flow Injection Mass Spectrometry

Open-access mass spectrometry[35−37] is the tool of choice for the chemist to prequalify their libraries for screening. The choice as to which protocol to follow is dictated in most part by the size of the library under consideration. Flow injection analysis (FIA) mass spectrometry is widely utilized by many groups for confirming the identity of expected products because it is the highest throughput and most easily automated analytical method. State-of-the-art autosampling methods permit samples to be analyzed every 25−30 s with a throughput of up to 2800 compounds per day. More recent parallel FIA-MS approaches[38] have enabled compound analysis throughput to increase roughly 4- to 8-fold. Shown in Figure 3A is the parallel FIA-MS analysis of a solution-phase compound library arrayed in microtiter plate format. Samples are introduced simultaneously to an array of injector valves, and the valves are sequentially rotated from load to inject to provide a rapid serial sampling of the samples. This method permits a microtiter plate of samples to be analyzed in as little as 12 min. Recently, Morand and coworkers[39] modified a Gilson 215 multiple probe autosampler to permit flow injection analysis of an entire microtiter plate of samples in less than 5 min, as shown in Figure 3B. Columns of a microtiter plate were sampled in as little as 10 s, offering the potential for characterizing an entire plate of samples in 2 min!

F. Fast Chromatography/Mass Spectrometry Analyses

LC/MS has fast emerged as the method of choice for the quality control assessment of spatially addressed libraries because the technique, unlike flow

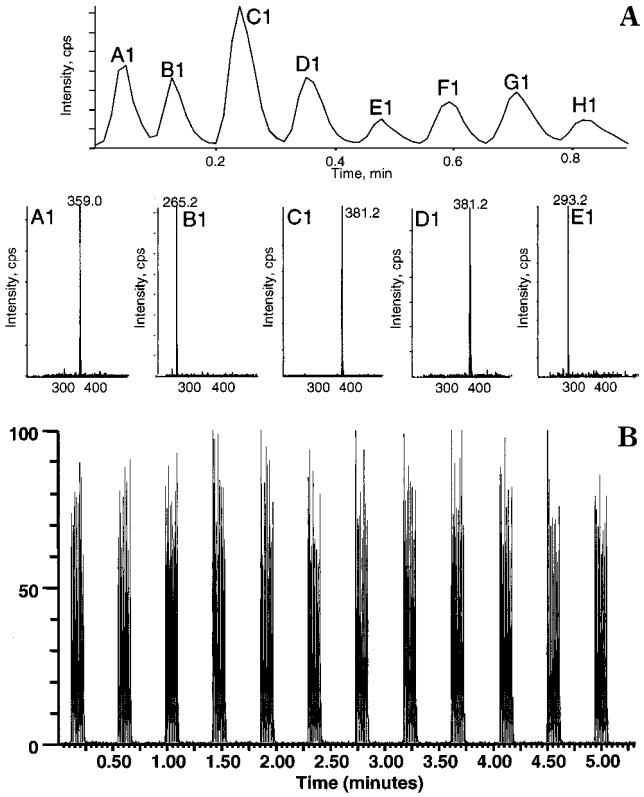


Figure 3. (A) Multiple probe flow injection analysis of eight samples arrayed down the column of a microtiter plate is achieved in nearly the same time it takes to autosample a single compound from a well of a microtiter plate using a conventional, single-probe autosampler. The eight samples were processed in a total of 1 min, giving rise to an "effective" analysis time of 7.5 s/sample. (B) Modified Gilson 215 permits ultrahigh-throughput flow injection analysis, permitting an entire plate of compounds to be profiled in less than 5 min. (Reprinted with permission from Morand, K. et al. 47th Conference on Mass Spectrometry and Allied Topics, Long Beach).

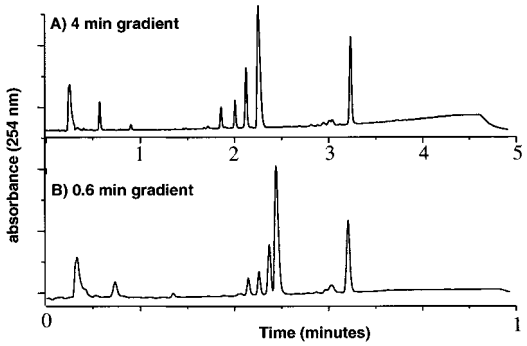


Figure 4. (A) Four-minute HPLC/MS separation of a crude product from a solution phase parallel synthesis library. A typical gradient profile for fast HPLC/MS compound library analysis is 10−90% acetonitrile in H_2O in 4 min with a 1 min equilibration time. (B) One-minute, pseudo-chromatographic separation of the same crude product from a solution phase parallel synthesis. (Reprinted with permission from Goetzinger, W. et al. *Am. Lab.* **1998**, *30* (11), 27−37.)

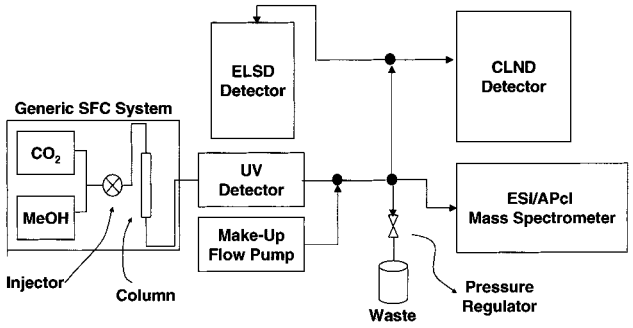


Figure 5. Typical SFC/MS system consists of a binary, high-pressure pumping system consisting of CO_2 and MeOH. The column, typically a diol or cyano column, is housed in a column oven to a temperature of 35−50 °C. A variety of flow-splitters (simple valco tees) are incorporated to divert the effluent flow stream to various detectors, such as evaporative light scattering (ELSD), chemiluminescence nitrogen (CLND), and mass spectrometer (MS). A pressure regulator is required to maintain a constant column outlet pressure.

injection mass spectrometry, provides the added measure of purity (and quantity) of the compound under investigation. In addition, "universal-like" HPLC gradients (e.g., 10−90% acetonitrile in water in 5 min) have been found to satisfy the separation requirements for the vast majority of combinatorial and parallel synthesis libraries. Fast HPLC/MS has been found to serve as good surrogate to conventional HPLC for assessing library quantity and purity.[40,41] Fast HPLC/MS is simple in concept. It involves the use of short columns (typically 4.6 mm i.d. × 30 mm in length) operated at elevated flow rates (typically 3−5 mL/min). An example of a fast LC/MS analysis of a combinatorial library component is shown in Figure 4A. Fast LC/MS run times incorporating these short columns is typically between 3 and 5 min including reequilibration. Recent reports by Li and co-workers[42] suggest that 'pseudo-chromatography' (in essence, step elution chromatography) in many instances provides a more rapid and reliable assessment of the quality of library synthesis than methods such as flow injection mass spectrometry. An example of a 1-min pseudo-chromatography LC/MS analysis is shown in Figure 4B.

More recently, investigators have begun evaluating complementary separation methods, such as supercritical fluid chromatography in combination with detection methods, such as mass spectrometry (SFC/MS), for compound libraries analysis. SFC/MS incorporates supercritical fluid CO_2 and a modifier, such as methanol, to facilitate separations. Under supercritical conditions, separations are carried out at linear velocities 3−5 times faster than LC/MS. In addition, SFC has been shown to be particularly well suited for the separation and analysis of both diastereomers and enantiomers. Recently, Greig et al.[43,44] and Wang et al.[45] showed that SFC/MS can be used in an analogous manner to HPLC/MS as a complementary tool for the analysis and quantification of compound libraries. An example of an SFC/MS system configuration is shown in Figure 5. The plumbing requirements for SFC/MS are, in general, more stringent than HPLC. Successful coupling with electrospray ionization or APCI is accomplished readily by using a long, highly restrictive peek tubing connector. To aid in compound ionization under conditions of electropsray ionization or atmospheric pressure ionization, practitioners in the field incorporate a third pump which delivers a methanol/water

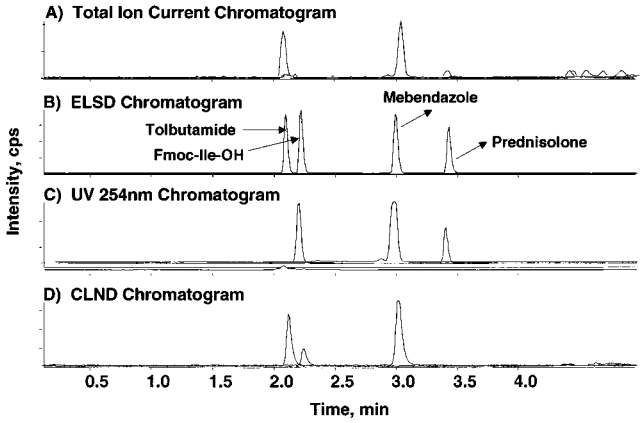


Figure 6. SFC/ELSD/CLND/MS analysis of an equimolar four-component mixture. The column flow rate was 5 mL/min. A portion of the column effluent was split to each of the three detectors (CLND, 200 μL/min; ELSD, 200 μL/min; MS, 100 μL/min). A makeup flow of 50/50 MeOH/H_2O (300 μL/min) was added to the flow stream diverted to the mass spectrometer ion source. Mass spectra were acquired using electrospray ionization with no special modifications to the ion source. (A) Total ion current chromatogram showing two of the four components ionize efficiently under electrospray ionization conditions. (B) ELSD chromatogram of the four components, all showing comparable response. (C) UV chromatogram (254 nm) shows some selectivity in detection as does (D) CLND detection.

solvent stream makeup flow to the mass spectrometer.

Orthogonal detection methods, such as chemiluminescence nitrogen detection (CLND)[46] and ELSD,[47,48] are purported to be more universal detection methods than UV are hence are being used with increasing frequency to assess reaction yields. CLND, as indicated from its name, measures the amount of nitrogen in a sample. In this method, compound is transferred to a high-temperature oxygen reaction chamber (set to 1000 °C), whereby the compound undergoes rapid decomposition to form nitrous oxide (NO). The liberated NO reacts with ozone (O_3) to form metastable NO_2, which is selectively detected by release of a photon, as shown in the scheme below:

$$\mathrm{R{-}N + O_2 \xrightarrow{950-1050\ ^\circ C} NO + \text{other oxides}}$$

$$\mathrm{NO + O_3 \rightarrow NO_2^*}$$

$$\mathrm{NO_2^* \rightarrow NO_2 + h\nu\ (600{-}900nm)}$$

CLND has been demonstrated to be a valuable tool for quantifying low quantities of material and has been shown to be particularly well suited to SFC-MS, for the principal reason that separations are carried out using solvents that do not contain nitrogen (i.e., CO_2 and CH_3OH). ELSD measures the mass (quantity) of the material directly, is often presented as being molecular weight independent, and is tool that has gained wide-scale acceptance for on-line quantification of compound libraries. An example of a separation of a four-component library analyzed by SFC/UV/ELSD/CLND/MS is shown in Figure 6. Using these various detectors, the chemist is able to obtain measures of purity of their library with greater confidence than when relying solely upon FIA-MS or LC/UV/MS data.

G. Purity Assessment of Compound Libraries

The issue of compound purity has received a great deal of attention in the past few years as more and more chemists are adopting high-throughput organic synthetic protocols but are unwilling to compromise the quality of the molecules submitted for biological testing. The general consensus target purity of a compound library compound before it is to be archived or screened for biological activity is between 85% and 90% (based on UV and or ELSD detection). The majority of mass spectrometry manufacturers now offer software packages that aid in the automatic determination of purity. Compound purity is typically determined by comparing the area under the curve for the component of interest relative to the sum of areas of all components in the sample, as shown in the equation below

$$\%\ \mathrm{Purity} = [\mathrm{I.A.}(\mathrm{UV}_{220})_{\mathrm{XIC}}/[\mathrm{I.A.}(\mathrm{UV}_{220})_1 + \mathrm{I.A.}(\mathrm{UV}_{220})_2 + \cdots + \mathrm{I.A.}(\mathrm{UV}_{220})_n] \times 100 \quad (1)$$

where the area under the curve for the expected compound is denoted by XIC and n represents the total number of peaks observed in the chromatogram. An example of automated purity assessment of a compound analyzed by LC/UV/MS is shown in Figure 7. In this example, purity is assessed at two different wavelengths, λ_{220} and λ_{254}. Macros (either visual basic or applescripts) are used for automated post-data acquisition processing, often producing hardcopies, graphical representations of the purity of a library as well as Excel files (text tab delimited) that summarize the purity results. For libraries generated in microtiter plate format, the results of each individual well may be color coded (or gray scaled) to reflect relative degrees of purity.[49] More often, as described earlier, compound purity is reported taking into account the purities determined from the UV, ELSD, and CLND detectors. In some instances, purity assessment has been made based on the intensity of the expected ion in the mass spectrum relative to the sum of the intensities of all ions in the spectrum. This method, however, is only a very crude estimate of purity, as ionization efficiencies for compounds can vary widely within and between classes of compounds. Though LC/MS (with UV and/or ELSD detection) has been adopted as the method of choice for assessing the quality and quantity of material prepared by parallel synthesis techniques, a decision still needs to be made as to what constitutes acceptable quality before submitting a sample for biological testing.

H. Purification Technologies for Combinatorial Chemistry

Historically, it was believed that solid-phase synthesis protocols eliminate the need for purification because excess reagents are removed readily by extensive washing. Unfortunately, even for solid-phase peptide synthesis, final products acid-cleaved

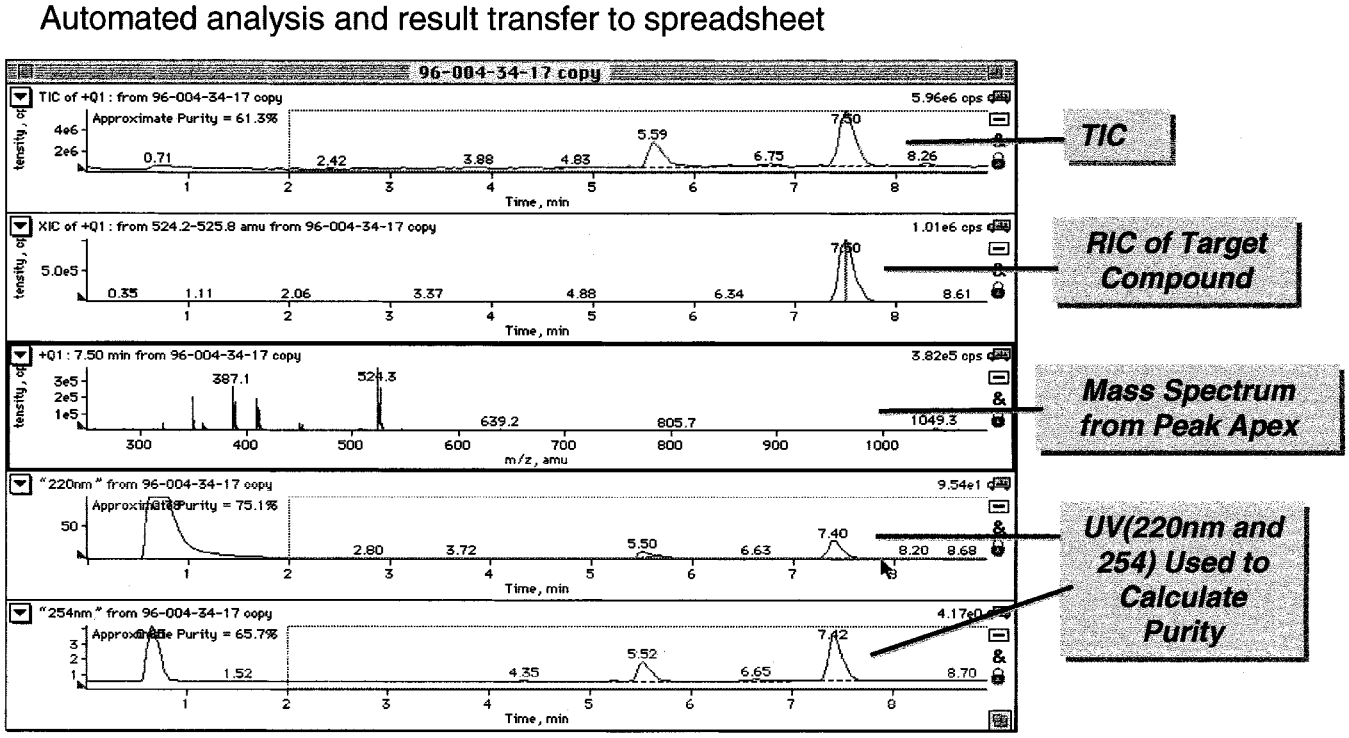


Figure 7. Purity assessment is a critical component in the decision process by the chemist as to whether their isolated compound is of sufficient quality to be submitted for compound registration and biological testing. To facilitate automated and rapid purity assessment of compound libraries, applescripts and visual basic scripts are used. (A) Total ion current chromatogram shows two components. (B) extracted ion chromatogram for the expected product identifies its retention time. (C) Mass spectrum observed for the expected product. (D) UV 220 nm chromatogram indicates the expected product is approximately 75% pure. (E) UV 254 nm chromatogram indicates the expected product is approximately 66% pure.

from the resin are found to be far from pure. Furthermore, parallel solution-phase synthesis has found greater popularity because it is readily automated and extends the "portfolio" of reactions available to the chemist for high-throughput parallel synthesis. The limitations with solid-phase synthesis and the movement toward parallel solution-phase synthesis are forcing numerous groups to evaluate and implement a variety of purification strategies.

A prevailing assumption within the medicinal chemistry field is that if the chemistry is sufficiently high yielding during the process development phase of synthesis, then it is reasonable to expect comparably high yields during the production phase of synthesis. In process development, a subset of the total library to be synthesized is rigorously optimized to maximize reaction yield. During production, it is assumed that all members of the library will behave similarly and that the desired product will be the major component in the well. The reality is that far too often the biological activity cannot be tracked to a single component or, in some instances, to the expected product in the well. Groups attempting to elucidate the active component(s) of the well have expended significant effort, only to find that the activity does not correlate to a single component within the sample. Consequently, more and more groups have embraced the value of "quality in, quality out" and are now applying the same analytical rigor to parallel synthesis chemical products as they have for more classical medicinal chemistry synthesis. These activities have enhanced the quality of structure−activity relationships (SAR) and structure−inactivity relationships (SIR) that can be derived from the assaying of these compounds for biological activity.

I. UV- and Mass-Directed Purification

Automated analytical techniques are now available to the chemist to perform high-throughput purification. Although HPLC has long been a method available to the chemist for product purification, only recently have these systems been designed for unattended and high-throughput operation. Weller and co-workers were one of the first groups to demonstrate "walk-up" high-throughput purification of parallel synthesis libraries based on HPLC and UV detection.[41,50] An open-architecture software interface enabled chemists to select the appropriate separation method from a pull-down menu and initiate an unattended automated reversed-phase UV-based fraction collection. Fractionation was achieved using a predetermined UV threshold. Multiple fraction collectors were daisy-chained in order to provide a sufficient footprint for fraction collection. Since the early work of Weller et al., a number of commercial systems have been introduced for walk-up preparative LC/UV purification (including Gilson, Hitachi, and Shimadzu to name a few).

Kibbey et al. made strides to streamline this process constructing a fully automated, more highly integrated system for preparative-scale purification of combinatorial libraries combining preparative HPLC (UV-based fractionation) and flow-injection mass spectrometry. In their method,[51] an analytical

LC/MS analysis was performed prior to preparative purification to identify the approximate retention time of the expected synthetic product. This information was uploaded to a stand-alone preparative LC system for final product purification. Fraction collection was initiated only during the window of time about the retention time of the compound (identified from the analytical LC/MS analysis) so as to reduce the number of fractions collected during the preparative HPLC analysis. Fractions were reanalyzed by FIA-MS to identify the mass of the collected fractions. Schultz et al. extended the preparative HPLC/UV purification method to perform separations in parallel, sampling from 96-well microtiter plates and collecting fractions directly into 48-well microtiter plates.[52]

At about the same time, the technique of preparative LC/MS was introduced. The mass spectrometer is used in this mode as a highly selective detector for mass-directed fractionation and isolation.[40,53−55] This technique provides a means for reducing dramatically the number of HPLC fractions collected per sample and virtually eliminates the need for post-purification analysis to determine the mass of the UV-fractionated compound. Preparative LC/MS is now widely incorporated in the pharmaceutical industry. Systems for preparative LC/MS are configured in numerous ways and are operated in numerous ways, including an expert user mode, walk-up or open access mode, or in a project team setting, supporting small teams of chemists working on similar chemistries. Two configurations of preparative LC/MS are shown in Figure 8. In Figure 8A, the instrument is configured in the prep-only mode, typical of many of the commercial systems available. All components of the system are under computer control and are hence truly automated. Important components of these systems are a 1:1000 flow splitter device and a solvent pump to deliver a methanol makeup flow to the mass spectrometer. The flow splitter and extra solvent pump serve the primary purpose of reducing the potential for overloading of sample into the ion source. A byproduct of the flow splitter and makeup pump is that it reduces the trifluoroacetic acid (ion pairing) in the ion source, which can affect the sensitivity of detection for acidic library components. Figure 8B shows a schematic of a system configured in an automated analytical/preparative mode of operation. In this configuration, the chemist is able to select between a variety of column sizes for either analytical, semipreparative, or preparative separations. The HPLC, switching valves, mass spectrometer, and fraction collector are under complete computer control.

An example of a mass-guided fractionation of a combinatorial library is shown in Figure 9A−C. In this example, the crude reaction product is only about 30% pure, as shown in Figure 9A. The component of interest shows a prominent single chromatographic peak when monitoring specifically for its corresponding mass (as shown in Figure 9B). Post-purification analysis of this singly isolated fraction (based on mass-directed fractionation) (shown in Figure 9C) demonstrates that the compound of interest was

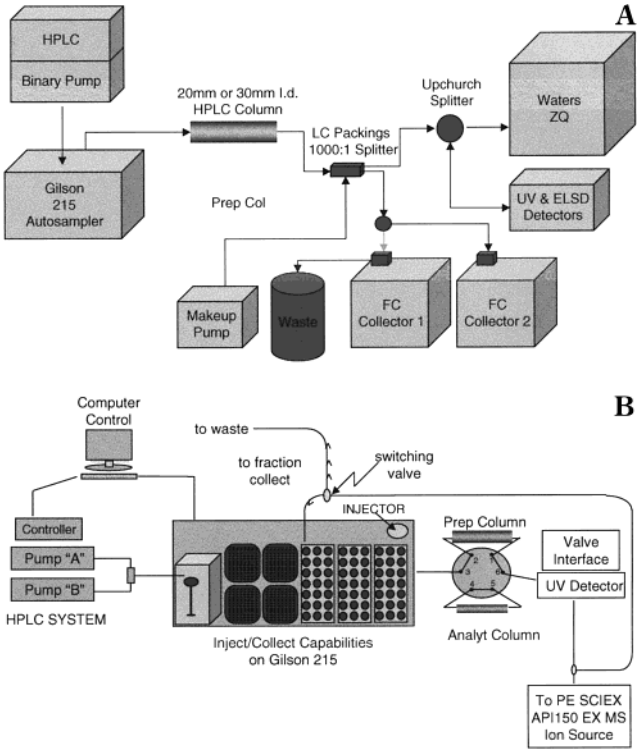


Figure 8. Schematic of automated mass spectrometry based purification systems. (A) Typical of many preparative LC/MS systems is the requirement of a high split ratio at the outlet of the column (1:1000) to divert only a small portion of the effluent flow stream to the mass spectrometer interface. A thrid pump is incorporated to facilitate transfer of sample to the ion source. Fractions are collected into dedicated fraction collectors. (B) Configuration of an automated analytical/preparative LC/MS system allowing for both autosampling and fraction collection on the same bed. A three-way valve, positioned over a fraction collector, receives a signal from the mass spectrometer as to when to fraction collect and when to divert to waste. Whereas UV threshold signals are used to trigger fraction collection for most preparative HPLC systems, mass-triggered fraction collection occurs when a target mass is observed in the mass spectrometer at a preset threshold level.

purified to greater than 90%. Had a UV-based fractionation system been used in this particular example, at least five individual fractions would have been isolated. Extending this to a 96-component library synthesized in microtiter plate format (and assuming this compound were representative of the quality of the members of the library), a UV-based approach would have led to approximately 400−500 fractions requiring reanalysis to locate the desired product. This would not only be a time-consuming reanalysis process but would require significant time to transfer the appropriate fractions to a screening plate for biological assessment.

Continued debate exists within the combinatorial chemistry community as to whether UV-based or mass-based fraction collection is the most appropriate tool for purifying compound libraries. The choice of technique probably should be governed by the relative importance of any given sample and the purification throughput requirements at any moment in time. As a simple rule of thumb, during the earlier stages of the discovery process, where large numbers and small quantities of compounds are being evalu-

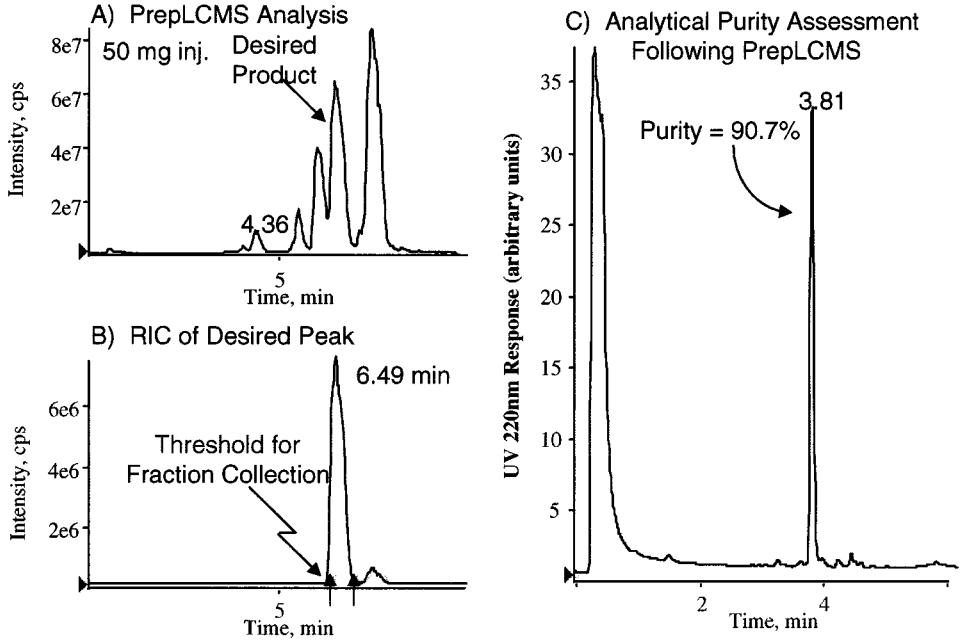


Figure 9. Fifty milligrams of a crude reaction product was solubilized in 1 mL of 50/50 MeOH/DMSO and injected onto a 20 mm × 50 mm i.d. reversed-phase column. Chromatographic separation was achieved using a gradient of 10−90% acetonitrile in H_2O in 7 min, following an initial hold at 10% acetontrile for 1 min to allow for removal of salts and low-retention materials. (A) TIC chromatogram shows five well-separated components. (B) Extracted ion chromatogram (XIC) for the expected product shows a single, prominent peak at 6.49 min. Fraction collection was initiated and terminated, as indicated by the arrows directly below the XIC peak. (C) Post-purification analysis of the isolated component shows the compound was purified to approximately 90% level.

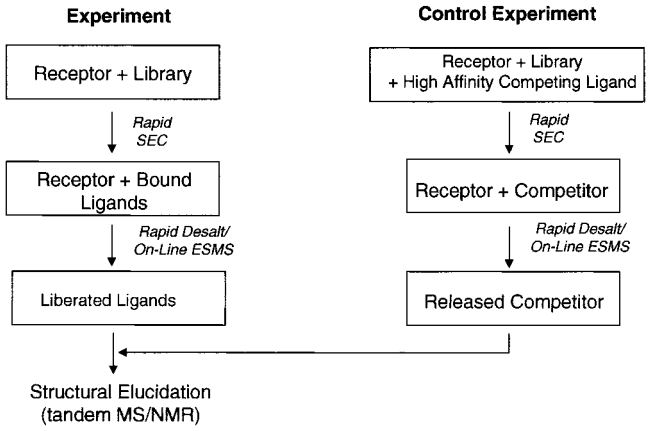
ated for biological activity, a mass-based fraction collection system may make most sense, since the total number of isolated fractions can be reduced to a minimum. At later stages of a discovery program (e.g., during late-stage lead optimization, where a smaller number and larger quantities of compounds are being evaluated for in vivo efficacy), UV-based method might likely take priority.

Independent of the debate, it is widely agreed that mass spectrometry serves as a highly sensitive and selective detector for analysis and purification of combinatorial libraries. Mass-triggered fraction collection enables compound libraries to be purified based solely on their expected product mass. Libraries can be purified maintaining a one compound−one fraction model, which facilitates sample tracking, registration, and biological testing and enables screening results to be readily correlated with synthetic structure.

III. Drug Screening by Mass Spectrometry

A number of mass spectrometry based approaches have been developed in order to identify leads from combinatorial libraries. Perhaps the most popular method to date has been to combine size-exclusion chromatography and mass spectrometry (SEC-MS). In this approach, compound libraries are incubated in solution with the target soluble receptor and then applied to the SEC column. Also referred to as "affinity selection mass spectrometry",[56] SEC enables free ligand (unbound combinatorial library components) to be separated from protein-bound ligands based on their interaction with a sorbent material which separates compounds based on molecular size. Eluted protein:ligand complexes are analyzed by

Scheme 1[a]



[a] Adapted with permission from ref 56. Copyright 1997.

tandem mass spectrometry (typically incorporating electrospray ionization) to "decode" the bound ligands based on their molecular mass and collisionally induced dissociation spectra. The process requires strict controls, some knowledge of "on" and "off" rates (i.e., knowledge of the kinetics of binding), and cooperation between mass spectrometrist and biologist to ensure that the optimal conditions to facilitate protein:ligand binding are compatible with mass spectrometric detection. A mass-based screening protocol is shown in Scheme 1.

Other groups have shown the power of mass spectrometry for identifying leads from combinatorial libraries by combining affinity chromatography, reversed-phase HPLC, and mass spectrometry.[57,58] In these multidimensional HPLC/MS methods, the target soluble receptor is immobilized to a solid support through a linker (e.g., via a histidine or biotinylation

tag). The combinatorial library is loaded onto the affinity column and incubated (typically at 4 °C) with the immobilized receptor for a defined period of time. Unbound ligands pass freely through the affinity column, and only the high-affinity ligands remain associated to the immobilized receptor. Bound ligands are eluted from the affinity column (either by competition with a known, high-affinity ligand, or by using high salt or acid solutions to disrupt binding) onto a desalting cartridge. The trapped ligands are then analyzed directly by mass spectrometry and their identities determined based on their unique molecular weight profiles. In an analogous manner, affinity capillary electrophoresis mass spectrometry and pulsed ultrafiltration mass spectrometry have been used for the separation and analysis of combinatorial libraries incubated with soluble receptors.[59,60] In an offshoot of these methods, Smith et al.[61] showed that protein:ligand complexes could be analyzed directly by electrospray ionization mass spectrometry. In their studies, a correlation between gas-phase protein:ligand complexation and binding affinity was observed. In each of these examples, the receptor is required to be isolated *and* soluble. Attempts to utilize this mass spectrometry based approach for identifying leads from combinatorial libraries probed against membrane-bound receptors (e.g., G-protein coupled receptors) has proved problematic.

The role of mass spectrometry as a screening tool for identifying hits or potential lead candidates from synthetic combinatorial libraries has more or less been confined to some rather elegant yet relatively small number of applications. This is in part attributable to the fact that mass spectrometry is an inherently serial technique and is not able to keep pace with the rapid advances made in ultrahigh-throughput screening methods where hundreds of thousands of compounds can be assayed per day in 384-well and 1536-well format. Nonetheless, clear opportunities remain for mass spectrometry as a screening tool. Split and mix libraries, provided they are designed appropriately to minimize molecular weight redundancy, are well suited to mass spectrometry-based screening strategies. A significant opportunity for mass spectrometry continues to be in the area of identifying hits and leads from endogenous sources of combinatorial libraries, such as natural product broths, extracts, and fermentations.

IV. Emerging Technologies

The synthetic throughput achievable by the medicinal chemist (adopting parallel synthesis strategies) has rendered analysis and purification one of the key (and possibly rate-limiting) steps in the discovery process. Although advances in sample analysis throughput have been clearly demonstrated, there is a limit as to how fast a separation and analysis can be achieved while maintaining good separation efficiency and quality analysis. Two techniques that are being developed to keep pace with the parallel synthesis revolution are rapid column switching and regeneration systems for enhanced

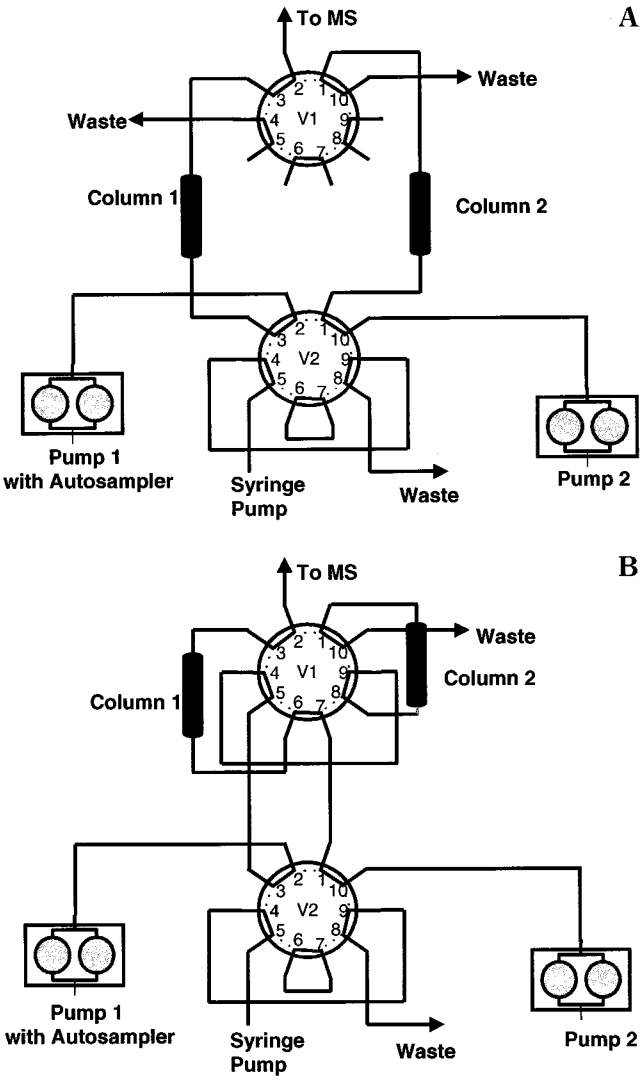


Figure 10. Rapid column switching system can be used to speed up serial based analyses. (A) Two 10-port switching valves are configured to allow for LC/MS analysis from column 1, while column 2 is in the equilibration mode. (B) The same set of 10-port switching valves, this time configured for gradient elution through column 1 while loading of the next sample is being accomplished on column 2. (Reprinted with permission from PE Biosystems, Framingham, MA.)

throughput serial-based analysis and parallel LC/MS methods. A simple and elegant modification of the LC/MS method is to incorporate a set of swtiching valves and a third pump to reduce cycle time between injections. While one column is being used to perform the LC/MS analysis, the other column is being regenerated, as described in a recent presentation by Chen et al.[62] An alternative use of 10-port switching valves is to allow for rapid serial sampling between columns, as illustrated in Figure 10A,B. This technique works extremely well for samples that are amenable to either isocratic or step elution. While one sample is being loaded onto one column, the contents of the other column are eluted into the ion source.

Di Biasi et al. and Wang et al. recently presented novel ion source interfaces enabling 4−8 samples to be processed in parallel, thereby increasing the sample analysis throughput dramatically over con-

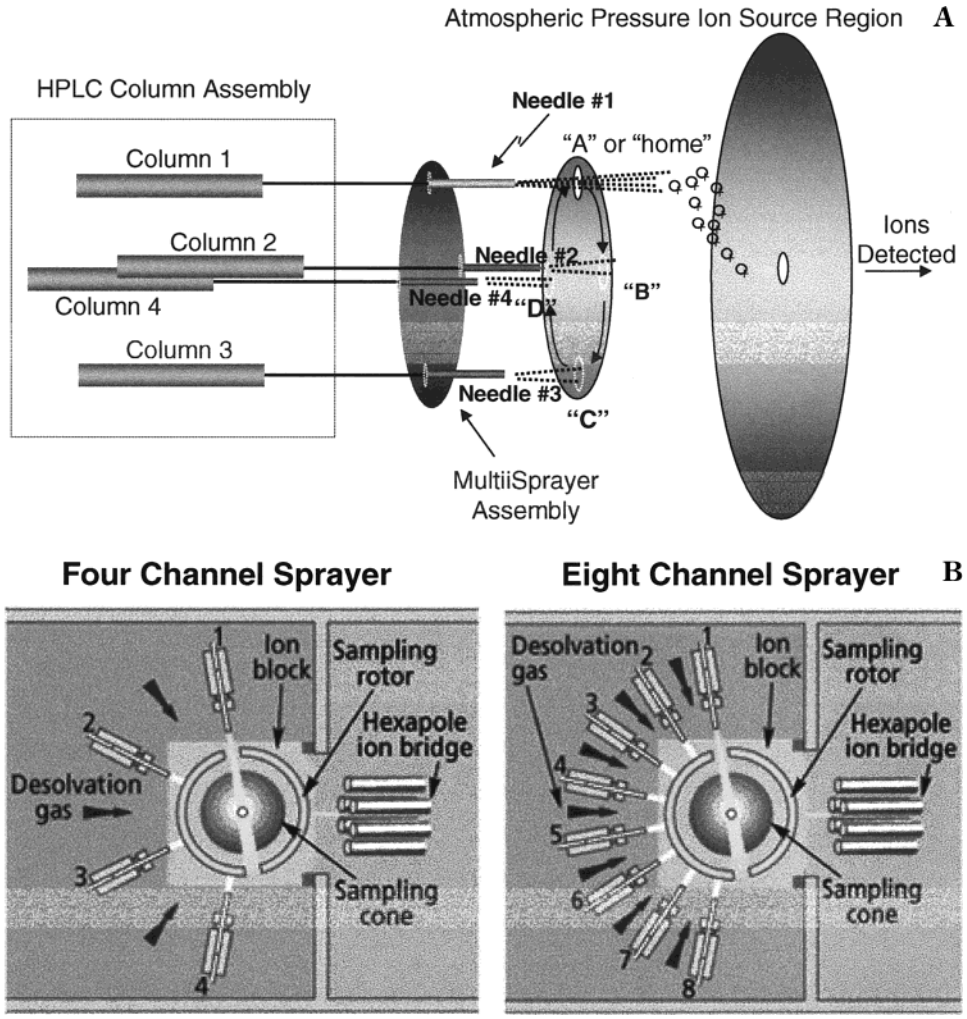


Figure 11. Multi-inlet ionspray source interface used to permit independent sampling of parallel fluid streams. (A) A four-head sprayer is mounted at the entrance aperture (orifice) of the mass spectrometer. A blocking device is positioned between the sprayer assembly and the orifice, permitting the effluent stream from sprayer 1 to pass through an entrance aperture of the mass spectrometer. (B) Illustration of a commercially available parallel spray sampling device. Typical intraspray rotation speed is 50 ms, and typical dwell/acquisition time at each spray position is 100 ms. Knowledge of the rotation and acquisition speed permits mass spectra to be readily deconvoluted.

ventional, serial-based LC/MS analyses.[63−66] Commercially available parallel spray interfaces consist of a multiple spray head assembly and a blocking device (e.g., rotating plate), enabling individual sprayers to be sampled at specific and defined time-intervals. Although the multiple sprays are delivered to the mass spectrometer simultaneously, they are sampled in a time-dependent manner, as shown in Figure 11A. A schematic of the commercially available unit is shown in Figure 11B. Minimum cross contamination is observed between channels, as shown in Figure 12.

A potential limitation to this approach is duty cycle. The duty cycle for an eight-channel blocking device is approximately 1.2 s (50 ms rotation time between each position of the spray assembly and 100 ms dwell/acquisition time at each of the spray positions). For combinatorial library LC/MS analysis, peak widths of 5−10 s for analytical runs and 10−30 s for preparative analyses are routinely observed. Thus, this relatively long duty cycle should have only marginal impact on combinatorial library analysis at present. Recently, Cole et al.[67] showed a variation on the multispray technique, incorporating a fast switching valve to "toggle" between chromatographic inlets. In this method, samples are introduced into a high-speed switching valve located outside of the ion source. Samples are sequentially transferred through the various valve positions, providing a temporally spaced flow streams into the ion source. Discussion of duty cycle and cross-contamination between channels were not made in this preliminary report.

To make parallel mass spectrometry more mainstream will require more effort on the chromatographic inlet side. Currently, many groups are taking the "poor man's" parallel purification approach, splitting the HPLC flow equally through a column array simply by aid of a flow splitter tee as shown in Figure 12. Some have referred to the technique of operating columns in parallel using one HPLC system as "split and pray" because the columns need to be maintained at nearly identical backpressures in order to maintain constant and identical flow through each of the columns. To achieve constant and identical flow through the array of parallel columns not only requires excellent quality control over column selection, but careful plumbing as well.

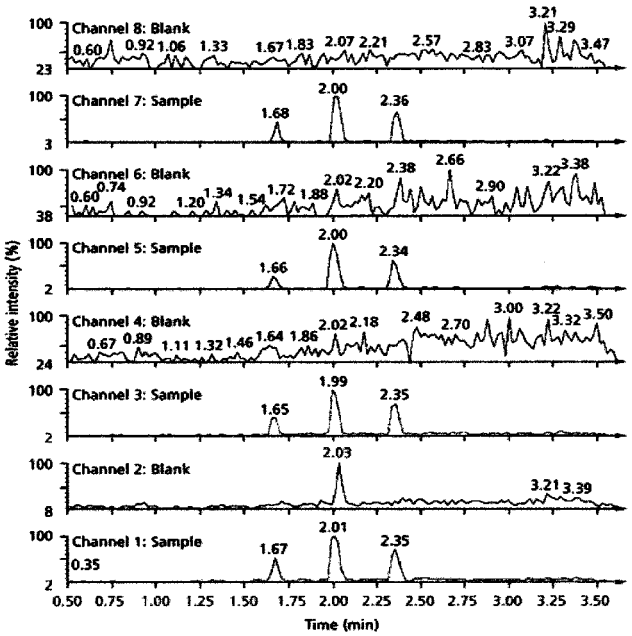


Figure 12. Standard mixture was introduced onto columns 1, 3, 5, and 7 and sampled, respectively, through sprayers 1, 3, 5, and 7. Blanks were introduced onto columns 2, 4, 6, and 8 and sampled, respectively, through sprayers 2, 4, 6, and 8. No cross-contamination was observed between adjacent channels. (Reprinted with permission from ref 65. Copyright 2000.)

Fortunately, concomitant to the advances being made in mass spectrometric detection of parallel flow streams are the advances being made in parallel sampling and parallel chromatographic separations. Coffey et al. pioneered a fully automated, high-throughput organic chemistry parallel purification system with parallel UV detection.[68] This innovation led to a dramatic rise in the number of parallel chromatography workstations now available for both intermediate and final product purification. Parallel chromatography−mass spectrometry systems now available commercially consist of multiple HPLC pumps, multiprobe autosamplers, parallel UV detection, and parallel mass spectrometer ion source interfaces. Instrument vendors are continually being challenged to introduce more cost-effective and more compact systems.

V. Conclusions

The role of mass spectrometry in drug discovery and combinatorial chemistry has clearly increased. Whereas only a handful of years ago chemists would "qualify" their compounds based on a TLC plate analyses, today mass spectrometry is now their preferred technique for compound identification and purity assessment. Whereas flow injection analysis was the primary tool for combinatorial library analysis only few years ago, today it is just one of many techniques available to the mass spectrometrist working in the field of combinatorial chemistry. The roles of mass spectrometry in combinatorial chemistry now span the continuum of early drug discovery from compound identification (e.g., high-throughput LC/MS), compound purification (e.g., high-throughput PrepLCMS), biological screening (e.g., affinity selection mass spectrometry), and high-throughput ADME profiling (e.g., high-throughput Caco-2 and cytochrome P450 assays).

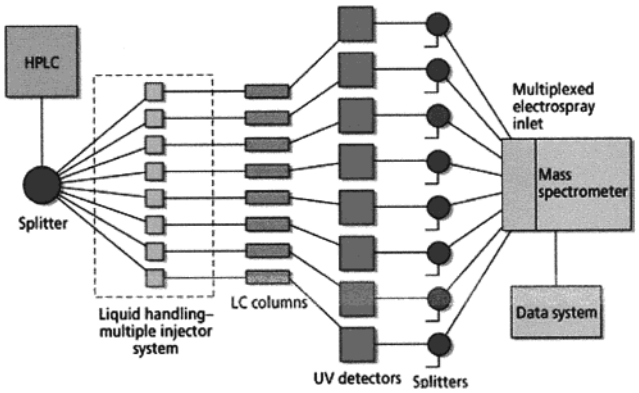


Figure 13. Schematic representation of parallel HPLC/MS system. Commercially available systems are configured with at least two sets of HPLC pumps to deliver equivalent flow to an array of HPLC columns, a multiple probe autosampler to deliver samples to the array of HPLC columns simultaneously, a multiplex UV detector, and an ion source interface to support independent sampling of simultaneously spraying samples. (Reprinted with permission from ref 65. Copyright 2000.)

Finally, parallel separations and parallel analysis are no longer concepts but have successfully captured the imagination of the mass spectrometry community. The question is not whether parallel technology will take hold within the mass spectrometry and analytical chemistry community, but only a question of when. In the opinion of this reviewer, the future of parallel analysis and purification is clear and it is indeed bright.

VI. Acknowledgments

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