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High-throughput preparative process utilizing three complementary chromatographic purification technologies

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Abstract

A high-throughput process was developed in which wells in plates generated from parallel synthesis are automatically channeled to an appropriate purification technique using analytical data as a guide. Samples are directed to either of three fundamentally different preparative techniques: HPLC with UV-triggered fraction collection, supercritical fluid chromatography (SFC) with UV-triggered fraction collection, or HPLC with MS-triggered fraction collection. Automated analysis of the analytical data identifies the product compound mass and creates work lists based on chromatographic properties exhibited in the data so that each preparative instrument cherry picks the appropriate list of samples to purify when a preparative-scale plate is loaded.

Keywords: Preparative chromatography; Supercritical fluid chromatography; Parallel synthesis; Instrumentation

1. Introduction

The primary goal of high-throughput combinatorial synthesis in the pharmaceutical industry is to provide drug-like compounds to high-throughput screening (HTS) [1–6]. Often the products of these syntheses are not adequately pure for biological screening since impurities can severely complicate interpretation of structure–activity relationship (SAR) data [4–6]. To provide libraries of highly pure compounds for screening, a purification process usually follows high-throughput library synthesis in the effort to accelerate the drug discovery process [7].

Modern purification techniques include preparative HPLC and preparative supercritical fluid chromatography (SFC) with UV or mass-directed fraction collection. Preparative HPLC with UV detection/triggering has a long history relative to SFC or mass-directed preparative techniques and is currently employed in industry for purification of combinatorial libraries [7–10]. UV-based preparative LC fails in cases where a sample is insoluble in water, where it elutes in the void with dimethyl sulfoxide (DMSO) or where the chromophore is too weak for triggering fraction collection.

Recently, mass-directed HPLC purification of compound libraries has accelerated in popularity due its capacity to produce single fractions based on the specific mass of interest [11–17]. It overcomes the limitations of poor chromophores and provides the capability to collect a product eluting in the void. Mass-directed purification fails in cases where a compound does not ionize to a degree exceeding the minimum threshold under the set MS source conditions. It also can fail for cases in which the compound detection timing relative to fraction collector triggering deviates due to a post-splitter clog (in this case it is possible to fail for every sample until the problem is discovered, a potentially disastrous error).

Preparative SFC exhibits many advantages over HPLC, and its application to combinatorial library purification has recently been reported [17–19]. In cases where solubility in aqueous mobile phase is poor or the compound is unretained using reversed-phase HPLC, UV-triggered preparative SFC can provide an effective alternative purification mode. Its limitations include cases in which samples are insoluble in methanol [20], contain highly polar solutes, and for

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compounds without chromophores. In our case, an attempt is made to purify every well synthesized using one of the three methods.

The process described in this paper details a high-throughput strategy to maximize the output of pure compounds available for screening by effectively utilizing a combination of these three purification techniques.

2. Experimental

The key to maximizing the rate of successful purifications of diverse library compounds is in generating appropriate analytical data. To this end, every combinatorial library well generated is analyzed by generalized high-throughput SFC–MS and/or LC–MS methods [21–27] specifically designed to facilitate automated SFC or HPLC purification based on electronic output from data analysis.

The analytical SFC–MS system used is a Berger FCM (Berger Instruments, Newark, DE, USA), interfaced to an Agilent MSD mass spectometer (Agilent Technologies, Palo Alto, CA, USA), as in Fig. 1A. Various SFC methods have been used for the purpose of high-throughput purification. The column was a 150 mm × 4.6 mm, 5 μm Zyrosil Pegasus, Zymor (Wayne, NJ, USA). The stationary phase for the Berger PrepSFC preparative system (see Fig. 1B) was the same but with column dimensions 150 mm × 21.2 mm. The analytical system flow rates ranged from 3 to 6 ml/min. Mobile phase compositions ramped from 5% up to 50 or 60% methanol in CO_2 at rates between 9 and 20% per min depending on the desired scaling relationship to the preparative SFC system and scope of the method being used. The preparative system usually operated with flow rates between 40 and 60 ml/min at 9% per min from 5% up to 50 or 60% methanol in CO_2 depending on the application.

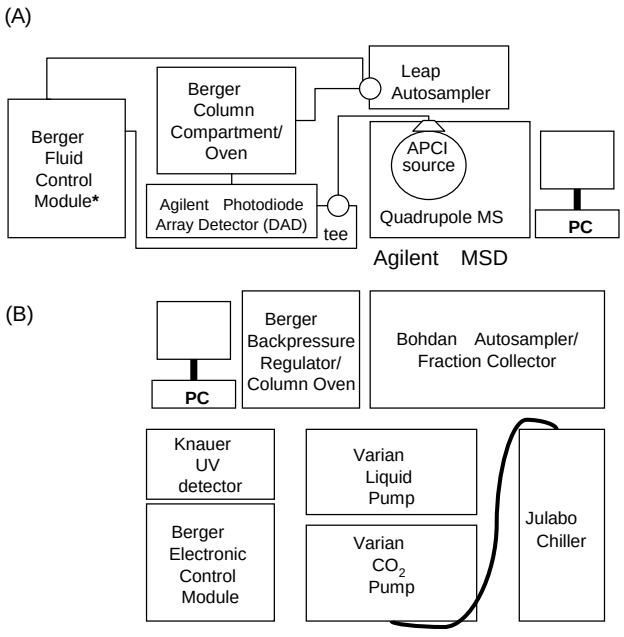

Fig. 1. (A) Analytical SFC–MS: block diagram of customized Berger/Agilent analytical SFC–MS system. (*) Fluid control module consists of CO_2 and liquid modifier pumps as well as backpressure regulator. (B) Preparative SFC system (BergerPrepSFC). PC: personal computer.

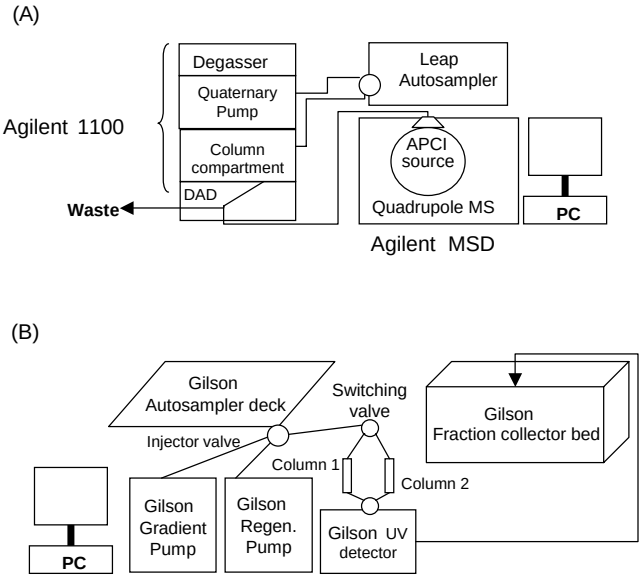

Fig. 2. Block diagram of: (A) Agilent analytical LC–MS system and (B) Gilson dual column preparative HPLC system.

The analytical LC–MS system was an Agilent 1100 LC–MSD as in Fig. 2A. The column generally used was a 50 mm × 4.6 mm, 5 μm Peeke Scientific Hi-Q C_{18}, while that for the Gilson Combinatorial Chromatography preparative HPLC system (see Fig. 2B) is the same but with dimensions 50 mm × 20 mm. The analytical system flow rates ranged from 1.0 to 2.5 ml/min depending on the desired scaling relationship to the preparative LC system and scope of the method being used. The preparative system usually operated with a total flow rate of 20–50 ml/min of acetonitrile–water with a constant 0.05% trifluoroacetic acid additive composition. A 0–100% linear gradient of acetonitrile in water was used with ramp rates up to 39% per min. The mass-directed preparative HPLC was a Waters FractionLynx system with dual 100 mm × 20 mm (5 μm) Peeke Scientific Hi-Q C_{18} columns. Flow rates of up to 50 ml/min acetonitrile–water were used on this system. Linear gradients from 5 to 100% acetonitrile in water (using 0.05% trifluoroacetic acid additive throughout) for durations of 4–7 min were used.

The analytical and preparative gradient chromatographic methods were defined such that that the gradient steepness parameter [28,29] is preserved. If this is true, then:

$$\left(\frac{Ft_G}{V_m}\right)_A = \left(\frac{Ft_G}{V_m}\right)_P$$

where F represents the flow rate, t_G the gradient time, and V_m the column void volume, respectively, for the analytical and preparative systems. For the procedures described here,

Table 1
Compared figures of merit among three major preparative purification technologies

System figure of merit for preparative mode	SFC–UV	HPLC–UV	HPLC–MS
Can purify compounds unretained on C_{18} (void elution)	×		×
Can purify compounds too polar to elute from np-column in CO_2–MeOH		×	×
Can purify compounds insoluble in H water but not MeOH	×		
Can purify compounds insoluble in MeOH but not H water		×	×
Can properly trigger collection of poorly-resolved species			×
Can purify poorly ionizing species	×	×	
Can purify weakly chromophoric species			×
Best chance to collect product in one fraction from complex separation			×
Excludes additive salts in the final product after facile evaporation	×		
Requires least evaporation time after purification	×		
Equipment price and maintenance costs reasonable (relative to others)		×	
Solvent and waste disposal costs reasonable (relative to others)	×		

Figures of merit advantages among either SFC–UV, HPLC–UV or HPLC–MS-triggered fraction collection purification systems. "×" Denotes the system(s) with the inherent advantage for each.

where the lengths and linear velocities of the analytical and preparative columns were identical, a good correlation resulted when preparative retention time was plotted against analytical retention time. As a result, once the systems were calibrated, the predictability of the preparative retention time based on the analytical method was very good, breaking down only with overloading and solubility differences on the preparative-scale. Note that this correlation applied for both HPLC and SFC purification schemes. The mass-directed preparative HPLC system by its nature did not require any correlation with an analytical scale gradient. Table 1 indicates which among the three preparative techniques in general exhibits an inherent advantage for some important figures of merit for chromatographic purification.

3. Results and discussion

3.1. Process flow

3.1.1. Process flow using UV-triggering for HPLC and SFC purification

In the process, a subset of monomers from a combinatorial library is analyzed by both SFC–MS and LC–MS, allowing the analyst to predict the best chromatographic mode for similar monomers when they appear in the full library. To illustrate this process, when samples begin their journey toward purification, those incorporating a given monomer are analyzed by LC–MS or SFC–MS as appropriate. Atmospheric pressure chemical ionization mass spectrometry (APCI-MS) is used with both LC–MS and SFC–MS due to its universality for compounds of interest and because of its compatibility with the flow rates used in high-throughput analysis [30–33].

Data from samples in 96-well plates are analyzed by either of the two techniques and are processed for the purpose of purification. The custom software identifies the product in the MS TIC (total ion chromatogram) signal, identifies the peak in the UV trace associated to this TIC peak, integrates the chromatograms and processes the resulting information. Each sample's integration data are recorded in text format (CSV), and queries incorporated into the data analysis program decide whether the well can be purified based on this information. UV area, baseline resolution and area percent are the major criteria used for selection. The output text file for an entire plate processed in this manner is composed of rows integration data for each well in a table, including a "yes" or "no" decision field for whether each well can be purified. This data, along with the predicted preparative-scale retention time windows for "yes" wells are written into an Oracle database. A custom program was written to create sample lists in the appropriate format for both the preparative SFC and preparative LC systems based on analytical information for a given plate. The "yes" wells are downloaded into the sample list along with their respective retention time windows for triggering UV fraction collection in the preparative system sequence table.

Finally, automated sample purification is initialized for any number of plates being queued on a system using a bar code scanner. Fractions are collected in pre-tared, bar-coded tubes in custom racks. An operator then scans the data rapidly using custom software utilities to decide and record electronically which tubes are kept or eliminated to ensure that a one-to-one correspondence exists between source well and tared tube containing the expected product. Generally, the vast majority of product fractions are contained in single tubes. The tubes are then evaporated using a GeneVac Megavap with a capacity for 676 tubes, and then reweighed using Bohdan robots with mass results written to the Oracle database. Gravimetric masses are associated with source samples in the database. The dried products are automatically reconstituted in DMSO with a Packard liquid handler, to a standard concentration based on the calculated net mass of product, then transferred into destination plates. An aliquot from each well in these product plates is transferred and diluted into an "analytical" plate which is subjected to quality control (QC) analysis by SFC–MS–evaporative

Table 2
Mass-directed purification's complements to UV-based purification

Reasons for "no" result	How our application of mass-directed purification overcomes
Inadequate baseline resolution	Longer column improves resolution, mass-specific triggering ignores resolution
Product elutes in void	Mass-specific triggering collects product even as it coelutes with DMSO
No product mass detected (APCI)	Possibility preparative method ESI signal strength is sufficient while analytical APCI signal weak
Product UV signal below threshold	Mass-specific triggering independent of strength of UV signal
Low product purity (area percent UV)	Mass-directed will collect product among impurities, some with sufficient weight/purity

Ways in which application of MS-directed purification adds to output from systems only capable of UV-triggered fraction collection. A "no" result from analytical data means no purification would be attempted without altering the UV-only "universal" high-throughput preparative method (hence abandoning the high-throughput process).

light scattering detection (ELSD) or LC–MS–ELSD as appropriate for the purification technique used.

3.1.2. Process flow modified to include MS-triggered purification

The initial viability assessment of library monomers by both SFC and LC (as previously described) directs individual wells on plates from the full library to the appropriate analytical method—SFC–MS or LC–MS. First, the wells from each plate thus chosen are analyzed by SFC–MS. Those wells on a given plate that pass analytical criteria for purification are queued for preparative SFC. Next, those wells from the plate chosen for LC–MS are analyzed. From these data, the set passing analytical criteria for purification are queued for preparative-LC.

In the purification step, those wells selected for preparative SFC are run first. Subsequently, the plate is moved to the preparative LC system deck.

There, those wells chosen by the LC–MS data for LC purification are attempted. Any wells on this plate not injected after completion of this process are then attempted on the mass-directed preparative LC system.

3.2. Benefits of the three purification modes applied

3.2.1. Benefits of the MS-directed purification system

Previously, any wells which were logged with a "no" decision for purification, were either not purified or were attempted on the complementary chromatographic technique with limited success. By contrast, initial application of the MS-directed preparative system forecasts a high degree of success even for wells with low product abundance in the sample. As a result we attempt to purify all "no" designated wells on this system. The Micromass ZQ mass spectrometer used on the Waters preparative system employs electrospray ionization (ESI) rather than APCI. This is used to enhance the likelihood for purification as many of the "no" results are due to insufficient product MS ion intensity in the analytical pre-purification analysis which uses APCI. A molecule that might not ionize efficiently or might fragment excessively by APCI may work well using ESI, alternatively. Furthermore, the predominance of only M + H ion in ESI spectra allows more accurate prediction of an efficient minimum intensity threshold for triggering fraction collection. Table 2 summarizes the ways that MS-directed purification complements our process to yield purifications from wells which would otherwise not be purified.

3.2.2. Benefits of the HPLC–UV purification system

Why then not use mass-triggered HPLC purification systems exclusively? In addition to the high degree of success we have experienced using LC and SFC without mass-directed fractionation, an inherent advantage to UV-triggered preparative systems is the in-line flow cell. The timing for fraction collection, dispensing from a valve into a tube triggered from detection of a minimum UV signal intensity threshold is a more direct procedure than that used in the mass-directed fraction collection configuration. In the latter configuration, sample flow through a splitter, then tubing, then an ESI inlet capillary precedes detection used for triggering. The timing delay relative to activating fraction dispensing is calibrated, but the possibility of clogs or restrictions in these lines affecting the timing in unattended operation exists, thereby putting the recovery of samples queued for purification at risk. In addition, the MS requires additional costs for set up and maintenance relative to a UV-only system. Even without the risk and cost factor, the HPLC system described enjoys additional high-throughput advantages over the mass-directed preparative system previously described. Though both HPLC purification systems so far discussed use dual-column technology, only the UV-triggered system is able to employ immediate column switching following product fraction collection. There is minimal delay from either the injection cycle or for the gradient continuation after a product elutes. The injector immediately aspirates the next sample and injects this new sample onto one column which was regenerated during the other column's gradient cycle. As applied, this nested process has performed purifications at a rate of <3 min per sample for tens of thousands of samples during the past year. The success rate for purifications on these systems is >90%, and recovery usually far exceeds 90% in the high-throughput mode.

3.2.3. Benefits of the SFC–UV purification system

Why then use SFC with UV-triggering for purification? SFC for semi-preparative purification enjoys a number of

advantages over preparative HPLC [20]. Some of these are listed below.

(1) Low viscosity and high diffusivity of supercritical fluids allow for efficient separations and fast column re-equilibration.
(2) The ability to change solvent strength by adjusting mobile phase composition, temperature and pressure for method development.
(3) Normal phase gives access to the nonpolar range of compounds reversed-phase HPLC does not separate well-HPLC and SFC complement each other.
(4) Separation in CO_2–methanol leaves fractions in methanol only—faster to drydown than ACN–water–acid.
(5) Exposure to acid or base additives as salts is avoided—accurate gravimetric mass for product.
(6) Sample integrity is protected by keeping close to neutral pH in drydown.
(7) Lower solvent and waste costs are realized.

SFC has been utilized in our laboratory with great success for high-throughput purification as tens of thousands of desired compounds of appropriate concentration and purity were recovered from combinatorial synthetic mixtures in the past year. Due to the ease of dry down and absence of salt forms of dried product, it is the preferred method of purification in our laboratory. It has relied on an accurate retention time correlation with analytical SFC–MS systems and realizes a success rate for purifications >90% while recoveries usually exceed 90% in the high-throughput mode.

3.3. Purity

Purity is determined from a post-preparative QC analysis of an aliquot of the final product well by LC–MS or SFC–MS depending on the chromatographic purification technique used [34]. Less than 10% of all purified samples over the past year have failed on the basis of low purity using the purification scheme described herein. Fig. 3 gives a chromatographic illustration of one sample's composition from crude through SFC purification, to final QC purity analysis of the product well by SFC–MS. In this example the product's purity improved from 55% initially to 96% after preparative SFC.

An example of the benefits realized with the integrated system set up with the intelligent inclusion of SFC, HPLC and mass-directed purification technologies is given in Fig. 4. Fig. 4A displays the output from the approach where all wells were purified using UV-triggered preparative HPLC, in which 52 of 88 wells recovered successfully. Fig. 4B and C illustrate the preparative output from the

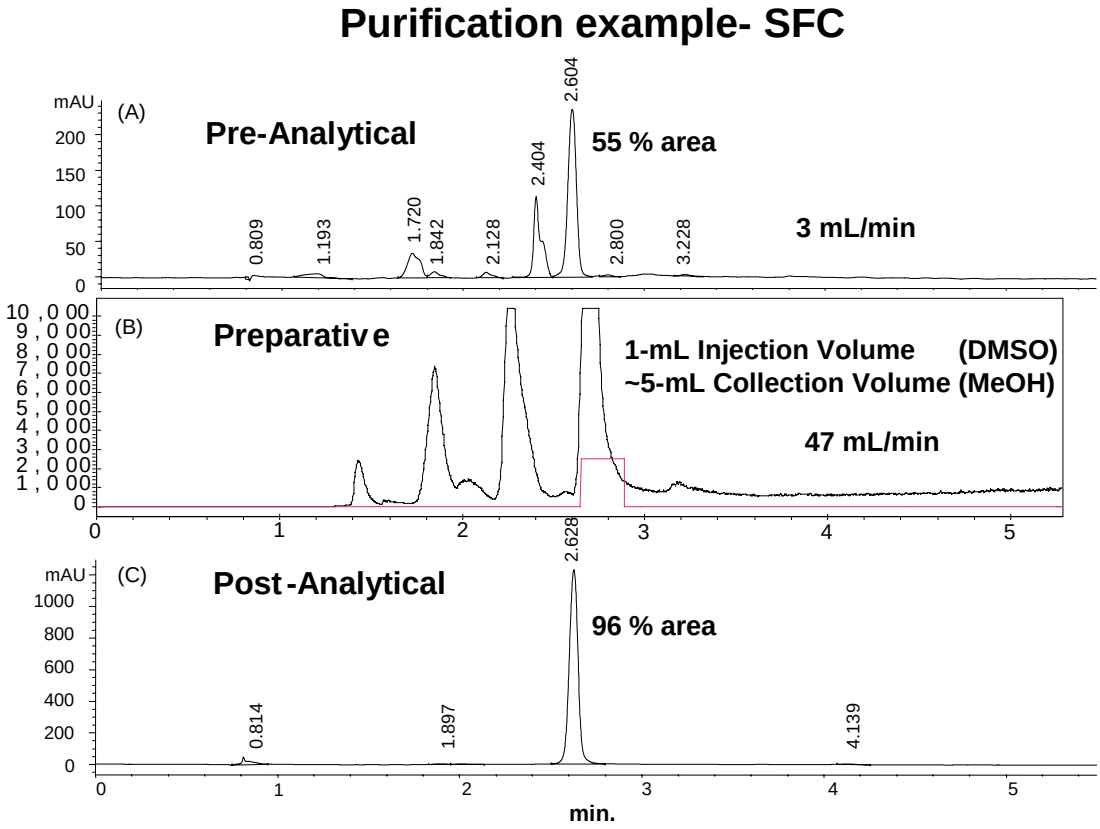

Fig. 3. Example of purity improvement through the preparative process based on SFC. (A) SFC–MS analytical data of crude sample—identifies product and specifies retention time window. (B) Preparative SFC run—rectangular trace represents time during which fraction collection occurred. (C) SFC–MS analytical data from purified well—product identified and integration gives percent purity.

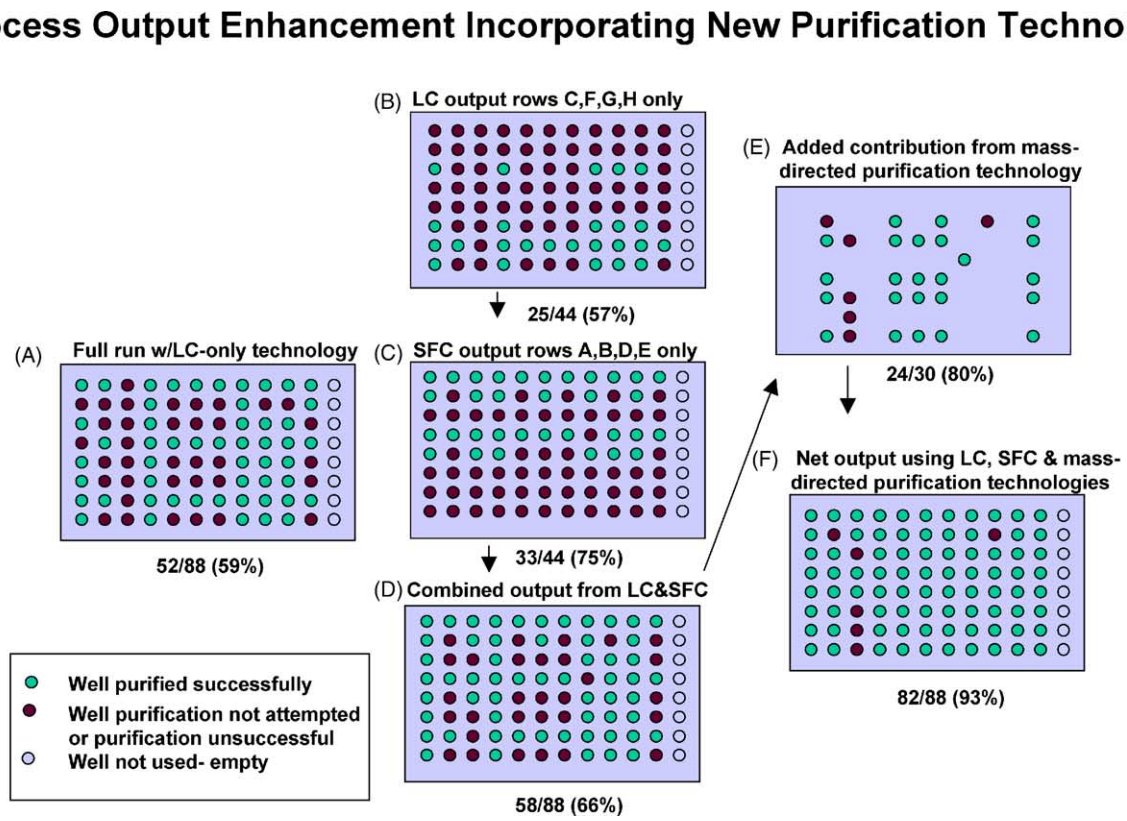

Fig. 4. Representation of output gain appending each purification technology. (A) Full output from preparative LC only; (B) rows C, F–H purified by LC; (C) rows A, B, D, E purified by SFC; (D) net LC/SFC output rows A–H; (E) mass-directed output for the 30 wells not run by LC or SFC; (F) net output including LC, SFC and mass-directed purification.

system in which both LC and SFC technologies with UV triggering were available. Rows C and F–H on the plate were run by LC since analytical data predicted LC would be able to purify more wells out of that set than SFC. Rows A, B, D and E were run by SFC since analytical data predicted SFC would be able to purify more wells out of that set than LC. Fig. 4D exhibits the benefit of combined output from using both SFC and LC purification to best advantage for this plate. The result was a total of 58 of 88 wells were recovered successfully. Next MS-triggered preparative capabilities were used for the 30 wells not recovered and shown in Fig. 4D. The plate representation in Fig. 4E illustrates only the remaining wells that were attempted by the MS-directed preparative system, i.e. not attempted by SFC or LC with UV triggering. The system was successful in purifying 24 out of the 30 wells attempted. Fig. 4F represents the combined output for this plate from the system combining all three preparative technologies. There were 82 successfully purified under this scenario compared with 58 for the SFC–LC combined system, or just 52 using UV-triggered HPLC technology alone.

The approach using three purification technologies was shown to maximize the output of the high-throughput purification process while taking full advantage of available preparative instrumentation capabilities.

4. Conclusion

The overall advantage to the system described consists in increased purification success rate for a structurally diverse set of small molecules as generated from combinatorial synthesis. The exact degree of benefit cannot be quantified as the precious products synthesized cannot be reproduced in quantity sufficient for such statistics. For the few sample sets subjected to this comparison, benefits of the degree described in Fig. 4 are not atypical. A purification laboratory equipped with these technologies is unencumbered by limitations previously described for systems based on only one preparative technology. An alternative system based on UV-only triggering is deficient in the ability to purify compounds without good chromophores. A preparative process based on MS detection only will usually collect the product unless it does not ionize sufficiently. If the MS-triggering threshold set too low, excessive collections will be triggered from the baseline and yield excessive fraction tubes with insignificant product concentration. Pre-preparative analysis for a preparative MS platform is still necessary not only to screen out compounds based on ionization efficiency, but also for poor separations. In these cases, a customized chromatographic method may be required which is difficult to incorporate in a high-throughput environment. By incorporating SFC, a normal-phase technique, into the system, it

is possible a separation adequate for purification would be achieved without a customized method. In addition, even when SFC does not achieve improved separation, systems based on HPLC purification alone miss the benefits of SFC purification for post-preparative processing: fast drydown and avoidance of the salt form of a product due to HPLC mobile phase buffers.

Only the process incorporating and integrating all three technologies will achieve the optimum success rate for mixtures of unknowns in high-throughput preparative chromatography. Our intent was to exploit the advantages and avoid the disadvantages of each by properly incorporating each system into our process flow. Key to the success of this scheme were effective analytical methods that scaled accurately to prep. Customized analytical data processing was also necessary to provide information as required to each vendor preparative system software platform. Integrating these essential components was a necessary achievement in the system design. The result is a streamlined process that successfully combines the highest possible purification throughput with the lowest possible attrition rate.

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