# High-throughput purification of compound libraries

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**Synthesis of combinatorial libraries by parallel synthesis, followed by high-throughput biological screening, is the new paradigm for drug discovery. Purity of these libraries is an important consideration to obtain high-quality assay data. Liquid–liquid extraction and solid-phase capture reagents are useful in special cases for small numbers of compounds. However, for libraries of a few thousand compounds, HPLC is a viable alternative. Beyond these numbers, factors such as solvent requirements, the number of fractions and tracking become prohibitive. Supercritical fluid chromatography has been successfully employed in automated purification instrumentation and is expected to be capable of purifying libraries of tens-of-thousands of compounds.**

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▼ The initial emphasis for producing large numbers of compounds focused on mixtures of compounds and was most thoroughly explored with peptides. This method took advantage of extensive experience with solid-phase synthesis of peptides by the Merrifield approach and combined it with the split-and-pool methods to provide compounds in extensive mixtures. Although providing a useful entry into the production of large numbers of compounds it had several drawbacks. Among these were the necessity for deconvolution to identify hits, the small quantities of the compounds prepared, the completeness or actual composition of the library, and the avidity problem with large numbers of weakly active compounds, which led to many false positive results.

The paradigm for high throughput chemistry has now shifted from large mixtures and millions of compounds made by split-and-pool methods, to the spatially dispersed, parallel synthesis of libraries in the range of a few thousands to tens of thousands of compounds. The output from the parallel synthesis approach is one target compound in milligram quantities per well of the synthesis plate. It is important to recognize that although a single compound is targeted, side reactions and unreacted starting materials frequently lead to impurities. Substantial efforts have been made to optimize reaction conditions to improve yields and select only those inputs that result in good yields in the crude reaction mixtures. Although partially successful, large libraries, in which only a fraction of the possible combination of inputs can be tested by initial optimizing methodology, usually result in a range of purity states. Additionally, to achieve reasonable purities of final products, substantial compromises frequently have to be made with regard to selection of inputs. Combinatorial libraries based on solution chemistry, rather than solid-phase chemistry, are even more problematic. Capture reagents might only be partially successful in removing excess reagents and will not remove neutral side products or other contaminants. Additionally, the nature of the capture reagents limits the choice of inputs incorporated into the products such that the capture reagents should not interact with these products as well as the excess reagents.

In contrast to the mixtures provided by split-and-pool methods, parallel synthesis is designed to produce individual compounds in single wells of a dispersed array. Because they are meant to be individual compounds rather than the mixtures historically attributed to ‘combinatorial chemistry’, they have often been referred to as ‘single, pure’ compounds. This is certainly true when compared with the situation with the split-and-pool mixtures but when compared with the purity state usually described for traditional, individually synthesized compounds, it definitely is not true. The confusion is in the use and misuse of the term ‘pure’ and equating it with intention rather than fact. With simple chemistries, such as

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coupling protocols used for amino acids to make peptides, in which reagents can be used in a large excess and then washed away, short reaction sequences can often be forced to completion and lead to relatively pure libraries. With the advent of much broader chemistries on solid-phase, coupled with the vagaries of synthetic chemistry, the situation has substantially changed. Frequently, when the nature of the inputs into a reaction is changed, the yield of product can be dramatically affected. To some extent this can be circumvented by careful selection of only those inputs that result in high yields but this puts a major constraint on the flexibility in the library design that one can achieve with any given library and inputs.

Finally, as the number of compounds that can be synthesized and assayed has dramatically increased, so has the time and effort required to characterize these libraries and to isolate and resynthesize active components. This problem, however, is compounded when the library is impure and HPLC-MS becomes necessary to locate and confirm the compound of interest.

## The case for purification

One view has suggested that, in the lead-generation phase, the objective is only to discover some biological activity in a compound, which can then serve as a starting point for further optimization, and that, therefore, compound purity is not necessarily an issue[1]. We, and others[2], believe that this viewpoint is problematic. The biological results on crude mixtures have led to false positives, missed active compounds present in low yields, time consuming resyntheses, as well as substantial efforts to confirm positive results. Unfortunately, the idea that a plate of systematically varied structures would lead to powerful structure–activity relationships (SARs) has largely been unrealized with libraries consisting of crude products in unknown amounts. To fully take advantage of the potential SAR information in a plate of compounds it is necessary to know the purity of the compounds, verify the molecular weight and quantify the amounts.

Another important, but more subtle, criticism of the current approach to the HTS of large compound libraries is that the focus is initially on potency and, possibly, selectivity. It is well known that other physical properties, such as solubility and membrane permeability, also have a major role in the successful development of a drug candidate. Traditionally, the decision to select a compound for further development was based on a compromise of potency and selectivity and the aforementioned other physical properties. With the current high throughput paradigm, lead selection is made only on the basis of potency and selectivity; optimization of other properties comes much later. Thus, in addition to screening for biological properties it would be highly desirable and advantageous to be able to measure selected physical properties that are important in the lead selection process, including solubility, lipophilicity, and membrane permeability. These can only realistically be measured, in a high throughput mode, with pure compounds. An intelligent choice of leads to optimize could then be made, based on a compromise of potency, selectivity and important drug-like physical properties.

If combinatorial chemistry is to fulfill its early promises and have a truly significant impact on drug discovery and successful development, then higher throughput automated analysis and purification methods must be developed. Pure libraries will permit useful SARs to be obtained at the initial stages of screening and will allow the measurement of physical properties known to be crucial for successful drug development.

## Potential approaches

Traditionally, purification of crude reaction mixtures has relied on a variety of techniques and more recently, polymer-supported electrophilic or nucleophilic capture reagents, or scavengers, have also been used. Fluorinated reagents have been proposed for work-up simplification. However, to be useful for the high-throughput purification of large numbers of compounds present in typical libraries, any purification scheme must be generally applicable for all chemistries and structural types, rapid, easily automated and capable of yielding pure compounds in a single pass. Although some of the above approaches have been investigated for their high throughput potential and have found applications in isolated, specific cases, few have met these generic high-throughput requirements.

## Liquid–liquid extraction (LLE)

Although liquid–liquid extraction has been extensively used in traditional synthetic chemistry, it is a difficult process to apply to combinatorial libraries. Emulsions, incomplete separations of layers, the organic versus water choice, all make automation difficult. Nevertheless there have been a few reports on the successful application of this approach[3–5].

An ingenious liquid–liquid or solid extractor has been described in which crude products are purified using aqueous extraction by partitioning the compounds, in 96-well plates, between aqueous and ethyl acetate layer[6]. After the layers separate, an extractor-pin assembly is inserted in each well reaching the aqueous phase, and following freezing of the aqueous layer, is used to remove the ice plugs (aqueous layer) from the wells; purities of 35–92% were achieved.

Automation of the LLE method has been described in which a 96-channel liquid–liquid handling workstation (Quadra 96) was used to load and bring into solution the organic extraction solvent and sample in the well of the 96-well sample plate[7]. An extraction plate, consisting of a 96-well plate fitted with a polyethylene bottom frit and filled with diatomaceous earth, was conditioned with an acidic aqueous solution. Solutions from the wells of the sample plate were transferred to the extraction plate where the partitioning between phases occurred. The organic layers were then eluted, under vacuum, into 96-well collection plates. The procedure required 15 minutes and, in selective cases, resulted in good recoveries and reasonable purities.

In a variation of the LLE approach, 'fluorous syntheses', suitable fluorocarbon groups, can be attached to organic molecules to render them soluble in fluorocarbon solvents[8,9]. Given that fluorocarbon solvents are often immiscible in organic solvents, mixtures can result in the fluorous-modified molecules partitioning out of an organic phase and into the fluorous phase in LLE. This has been suggested to be a potentially useful approach in selective cases, for the purification of parallel libraries.

### Solid-phase extraction and capture reagents (SPE)

In solution-based combinatorial libraries, because the product is not on solid support, excess reagents cannot be directly removed by washing as they are in solid-phase chemistry. Purification of these libraries has relied on the development and use of polymer-supported electrophilic or nucleophilic scavenger agents. Although readily automated, these procedures are not general purification procedures but rather are designed for a specific reagent removal. Additionally, there can be no functional groups in the products (e.g. acidic or basic groups) that could react with the scavengers. Although somewhat restricted in their application, this scavenger approach has nevertheless been successful in several cases. For example, polymer-supported aminomethyl resin was used to scavenge excess isocyanate, acid chloride and sulfonyl reagents[10]. The resulting acylated resin was then removed by filtration to leave neutral products behind in solution. Alternatively, polymer-supported isocyanate, aldehyde and benzoyl chloride groups can be used to scavenge excess amine reagents. These approaches were used in the synthesis of secondary amines, amides, ureas, thioureas, sulfonamides and carbamates.

An interesting variation on this approach describes the strategy of purification of a library of galactose derivatives[11]. In this case, purification was accomplished by protecting the 2-, 3-, 4- and 6-hydroxyl groups of a galactose derivative as their hydrophobic *O*-laurates. The highly hydrophobic multiple *O*-laurates were adsorbed onto C18 silica and excess reagents and byproducts from the synthesis were washed away with methanol. Products were eluted with pentane. There have also been reports using a combination of solution and solid-phase extractions to purify combinatorial reaction mixtures. In this case, excess electrophiles used in the synthesis were quenched with 2-mercaptoethanesulfonic acid sodium salt[12]. Upon workup, the quenched reagents partitioned into the organic layer as thioether sufonates. The SPE approach has also been used to separate regioisomers[13]. In the high throughput synthesis of 2-amino-6-alkylpurines, filtration through an acidic alumina pad or scavenging by AG/Dowex-50W-X8 resin provided diverse N9 regioisomers selectively in moderate yields with purities >90%. This method was used in a high throughput format for the purification of a 6,9-disubstituted purine library.

Finally, an interesting variation of this approach uses a resin reagent to capture the product, which is then purified by washing away the soluble excess reagents[14]. The resin-bound product can then be further modified by solid-phase synthesis.

Both anion and cation exchange chromatography have been used extensively as a route to library purification. For example, anion exchange resins have been used to efficiently remove carboxylic acid by-products and cation exchange resins to remove excess amines[15–18]. Several reviews have recently been published on the use of solid-phase extraction[19–23].

### HPLC

For more general approaches to library purifications, HPLC has received the most attention because of its adaptability to automation and the widespread experience with this instrumentation for separations of a variety of structural types. Traditionally, commercial HPLC systems have been primarily used for repetitive purification of sequential batches of the same sample of which the chromatographic properties were well understood. By contrast, high-throughput purifications must deal with samples for which there is no prior knowledge of the chromatographic properties and therefore must rely on 'universal' gradients and peak detection devices. Furthermore, in addition to automatic sample injections, there is a necessity for collection devices that permit adequate fraction collection, both in terms of potential numbers of fractions, as well as capacity when the number and quantities of mixture components are unknown. Although not an issue with small numbers of compounds, when the library to be purified consists of tens of thousands of crude reaction mixtures, each with three to five components, the number of fractions that need to be collected, analyzed, combined and tracked is enormous.

Recognizing the above issues and building on existing instrumentation, a few commercial high-throughput, semi-preparative HPLCs have been developed along with reports of several 'in-house' approaches.

The Biotage Parallax™ system (Dyax, Charlottesville, VA, USA) has been described in detail[24–26]. The system can purify ~75–100 mg crude reaction mixture. The crude reaction plates are subjected to high throughput HPLC purification and multiple fractions for each sample are collected in tared tubes (18 ml capacity). Peaks are detected at two of four possible wavelengths (219, 254, 280 and 307 nm) and this triggers fraction collection. Fraction collection is triggered above a predetermined threshold to avoid collecting minor peaks. Once the chromatographic purification of the plate is complete, the collected fractions are subjected to MS analysis and evaporative light-scattering to identify compounds and determine approximate mass. After the data analysis, the fractions containing the target compound are collected together and evaporated. The system can provide up to 600 fractionations a day. Four samples can be handled in parallel using the same gradient. Standard reverse-phase binary gradients of acetonitrile, or methanol, and water are employed. Each injected sample is chromatographed on a dedicated column and collected in a dedicated fraction collector. Flow rates are 30 ml $min^{-1}$ for 5.5 minutes followed by a wash of 100% organic for 1.5 minutes at 35 ml $min^{-1}$ then equilibrating at starting conditions and holding for 2 minutes (35 ml $min^{-1}$). As will be discussed later, these parameters have a significant practical impact on the amount of solvent used for library purification and illustrate the problems when dealing with large numbers of individual compounds. The collected samples are analyzed by electrospray or atmospheric chemical ionization–MS (ESI–MS or APCI–MS). This requires ~50 sec $sample^{-1}$ to give 72 peaks $hour^{-1}$. A typical daily output for the purification process is 288 samples per instrument. Because of the number of fractions and post purification steps, data tracking is an important issue.

Several 'in house' semi-preparative, high-throughput HPLC systems have been described. For example, researchers at Bristol Myers Squibb (Princeton, NJ, USA)[27] describe a six channel system that handles ~40 mg of crude reaction mixture for each injection. Peaks are detected using UV at 220 nm and gradients for preparative work are run at 20 ml $min^{-1}$ with 20 x 50 mm columns and a 10 minute runtime. Cycle times were ~7 minutes and a theoretical maximum of 200 samples could be done in 24 h. Collection is accomplished with a six-position multiport valve that directs the eluant flow path to six fraction collections connected in parallel. This provides a total fraction collection capacity of 384 tubes of ~38 ml each.

CombiChem (now Dupont) (San Diego, CA, USA) has described an automated HPLC–MS system for high throughput purifications (Refs 28,29; Kassel, D.B. *American Society of Mass Spectrometry Fall Workshop on Combinatorial Chemistry, October, 1997, San Diego, CA, USA*). This automated HPLC–MS incorporates fast, reversed-phase C18 HPLC (5–10 min analysis time) and ESI–MS. Instead of using UV, the method uses real-time MS ion signals to trigger fraction collection and only the mass of the compounds of interest are collected. This format avoids the issue of large fraction collector arrays and eliminates the need for post-purification analysis or pooling of fractions collected.

Pfizer (Sandwich, UK) and Micromass (Manchester, UK) have codeveloped a workstation in which a combination of UV absorbance and MS data are used to make real-time decisions for HPLC fraction collection (Ref. 2; Kiplinger, J.C. *et al. Pitticon '97, March, 1997, Atlanta, GA, USA*). Parke-Davis (Ann Arbor, MI, USA) has reported on an automated semi-preparative HPLC (Ref. 30). This system appears to rely on analytical analyses to set the appropriate fraction collection window for the preparative run. The Abbot Laboratories group (Abbot Park, IL, USA) has also reported on preliminary efforts for automated HPLC purifications[31]. Figure 1 outlines the various strategies that have been employed in high throughput HPLC instrumentation for the purification of compound libraries.

## Supercritical fluid chromatography (SFC)

HPLC is an effective library purification method for libraries of a few thousand compounds, but as the number of compounds increases to tens of thousands, several practical issues become substantial limiting factors. After an HPLC chromatographic step is accomplished, the mixed solvents must be removed from the collected peaks, fractions are combined (usually 3–10; when LC–MS is not used) and subjected to a time-consuming evaporation and lyophilization process. HPLC-grade solvents can be expensive and disposal

**Table 1. Comparison of solvent requirements for HPLC vs SFC**

| | Number of compounds | Solvent requirement[a] Liters (tons) | Time to purify[b] (11 hr day) |
|---|---|---|---|
| HPLC | 20,000 | 5763 L (6.3 tons) | 764 hrs (69 days) |
| SFC | 20,000 | 120 L | 500 hrs (45 days) |

[a]Calculated based on reported flow rates for semi-prep HPLC (~30–35 ml $min^{-1}$), the time for chromatographic separations (~9 min) (Refs 24,27).
[b]Assuming ~288 compounds per day (Ref. 24).

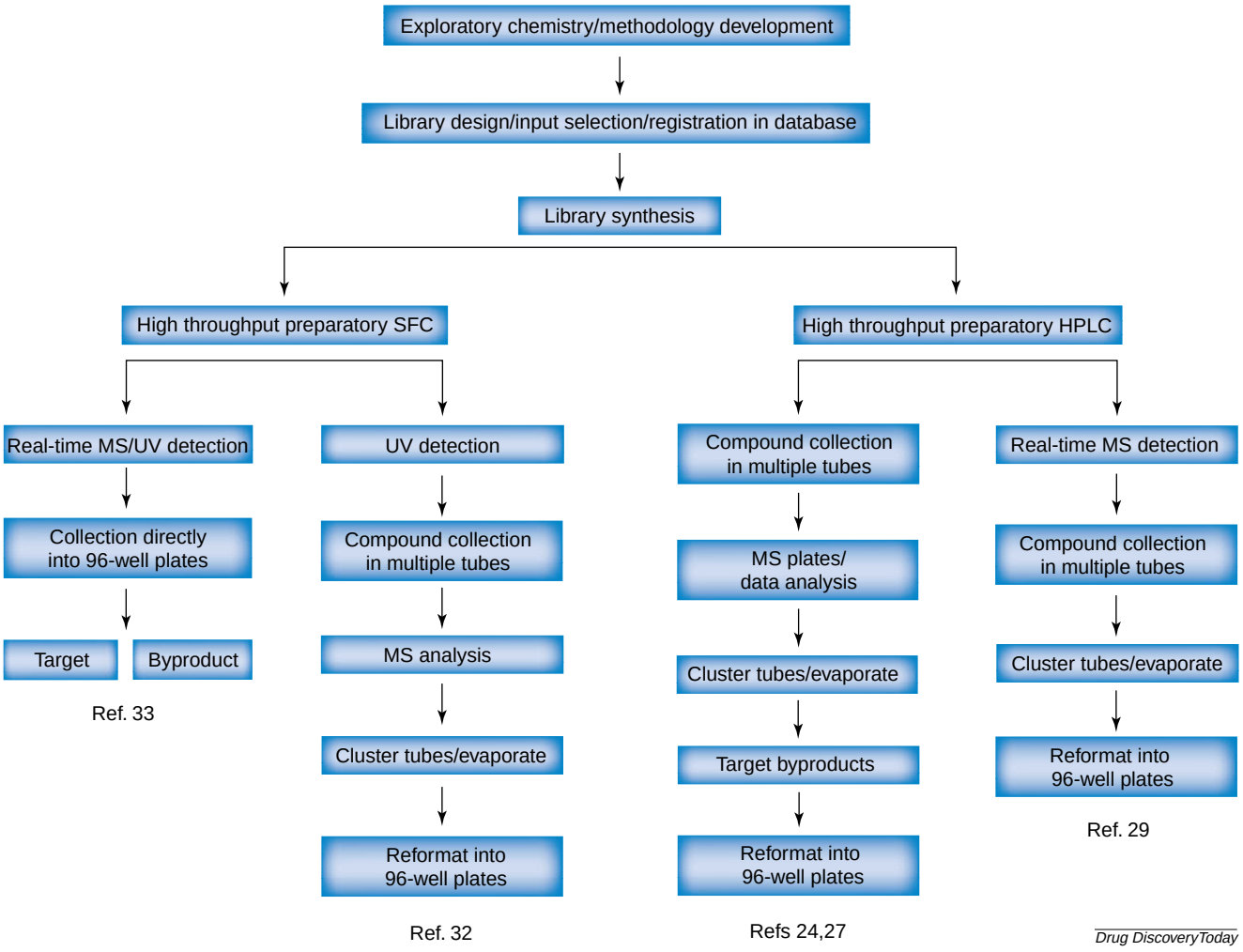


**Figure 1.** The figure shows comparative strategies for high throughput purification of parallel synthesized compound libraries utilizing supercritical fluid chromatography (SFC), or HPLC. Two collection strategies are shown for SFC, one a real-time MS directed one-to-one mapping from crude plates to 96-well collection plates and the second a method of collection into tubes that requires post-chromatographic identification and reformatting into 96-well plates. The HPLC arm shows two current collection strategies, one with real-time target identification by MS before collection and the second a collection of each peak and post analysis MS for identification.

costs can exceed the original purchase prices. Estimates can be made for solvent consumption of libraries of the order of tens of thousands and with regard to solvent purchase and disposal, these costs can be staggering (Table 1).

To address the limiting factors described above, an alternative to HPLC has been explored with supercritical chromatography[32–35](Fig. 1). Supercritical fluid chromatography (SFC) is typically employed as a variation of normal-phase HPLC but one in which a compressed supercritical gas (e.g. carbon dioxide) is used as the mobile phase. This confers several advantages. Using SFC, higher flow rates and longer columns are possible; SFC doesn't suffer the back-pressure problems present in organic-solvent-based HPLC. Importantly, in SFC the mobile phase immediately 'evaporates' when the external pressure is removed. Carbon dioxide is relatively safe, readily obtainable, inexpensive and available in pure form. It is a non-polar solvent that requires the addition of a polar modifier, often methanol, to increase its polarity, solvent strength and general chromatographic suitability. Analytical SFC has demonstrated the capabilities of this approach to separate drug-like compounds and it has been shown to be comparable to HPLC in this regard[32,36].

If a polar modifier is used as part of the mobile phase, when the pressure drops, two phases form. One phase consists of polar modifier and eluted solutes and the other is predominately gaseous carbon dioxide. The challenge for a useful SFC semi-preparative instrument is to efficiently collect the liquid phase with the desired compound and to vent the carbon dioxide. Given that the expansion can be 500:1, a major technical challenge has been to contain the resulting

gas–solvent mixture and allow the separation to take place. Various strategies can be employed to collect this gas–liquid mixture including trapping it in a column of liquid, using various types of packed beds with high surface areas, and collecting with specifically designed apparatuses. One solution[32] is to do the collection in 50 ml test tubes. In this case, the collection cassette allows multiple fractions to be collected from each sample. Collection efficiency is high and carryover is minimized. The resulting samples must then be identified using MS and then reformatted into plates for HTS.

Ontogen (Carlsbad, CA, USA) has taken an alternative approach[33]. The company designed a four-channel instrument that allows four plates to be simultaneously purified. The crude mixtures from each well are automatically injected and subjected to SFC chromatography. Eluted peaks are first detected using UV with a diode array detector (one per channel) and fraction collection is triggered on-the-fly by a time-of-flight MS. A special 'splitter' device allows the flow of each of the four channels to be sampled by diverting a small portion of the flow from the UV-identified peak to a single MS. This is accomplished without inter- or intra-channel contamination. Flow rates are 12 ml $min^{-1}$ with a gradient time of 5.5 minutes (2–35% methanol) followed by a 0.5 min wash. On target mass confirmation, fraction collection is triggered by the MS to direct the peak flow either into a target plate or a by-product plate. A specially designed fraction collector allows collection directly into two 2 ml deep-well microtiter plates, one for the target compounds and the other for the by-products, with well-to-well mapping with respect to the input plate. Small, disposable 'expansion chambers' are used in the collector and are replaced for each well. This facilitates sample recovery and avoids carryover and well-to-well contamination. Target peaks are collected up to 31 seconds (approximately equivalent to a volume of 1.5 ml), which is greater than the typical peak width times for the employed conditions, allowing for complete sample recovery. This then avoids the collection of multiple fractions and the subsequent reformatting and tracking necessary in other systems. The system allows for a maximum purification of 384 samples in a 10-hour day. After purification is complete, the 96-well collection plates containing methanol are evaporated down to yield dry product.

In both HPLC and SFC chromatography the achievable purity for any given sample, unless there is a baseline separation of the peaks, will of necessity be a compromise between desired purity and the amount of material recovered. This will be dependent on the degree of overlap of impurity peaks with the desired compound and the peak selection algorithm. For the Ontogen system, the criteria used was to obtain at least 85% purity for moderately overlapping peaks and 100% for baseline separation.

## Summary

Several strategies have been utilized for the purification of parallel synthesized libraries. Some of these are based on liquid–liquid extraction and solid-phase capture reagents are useful in special cases for relatively small numbers of compounds. To achieve purifications comparable to that achieved for traditional one-at-a-time syntheses, chromatographic separations are necessary. HPLC is a viable alternative for libraries of a few thousand compounds but for more compounds practical considerations of solvent requirements, number of fractions collected and tracking become prohibitive. SFC addresses these issues and has been successfully employed in automated purification instrumentation to purify libraries in the tens of thousands of compounds.

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