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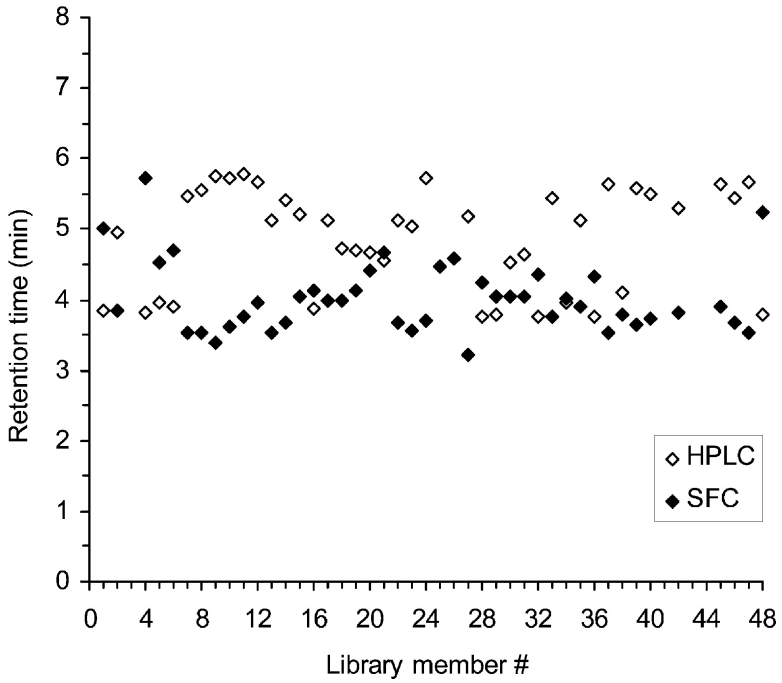

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Comparison of Preparative HPLC/MS and Preparative SFC Techniques for the High-Throughput Purification of Compound Libraries

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A diverse set of 16 high-throughput organic synthesis libraries, consisting of 48 samples per library, has been purified by both preparative supercritical fluid chromatography (SFC) and preparative high-performance liquid chromatography (HPLC). This paper details the relative effectiveness of these two purification techniques in terms of success, yield, and purity of final product.

Introduction

The increasing use of parallel synthesis and the successful application of automation to synthetic chemistry have created a demand for high-throughput purification of compound libraries. The drug discovery process relies upon a large number of new compounds for biological screening, and it is recognized that the quality of these compounds is of paramount importance. High-purity, fully characterized compounds are required in order to give reliable structure activity relationships and minimize false positives/negatives from biological screening data.[1]

Compounds of acceptable purity may be obtained through the adoption of new reaction workup strategies, such as the use of solid-phase extraction,[2,3] liquid−liquid extraction,[4,5] resin-bound scavenger reagents,[2,6] or fluorous extraction.[7,8] When reaction conditions make these methods unsuitable or the compounds obtained are not of high enough purity, reversed-phase high-performance liquid chromatography (HPLC) is often the technique of choice. Purification of libraries by preparative HPLC has evolved from using UV-triggered fraction collection[9] and subsequent analysis of fractions to mass-directed fraction collection using online liquid chromatography/mass spectrometry (LC/MS) to collect fractions only when the mass of the compound of interest is detected.[10−12] Further improvements in throughput have been achieved by purifying multiple samples in parallel, whether by systems using UV-triggered fraction collection[13] or mass-directed fractionation through the use of mass spectrometers equipped with multiplexed ion sources.[14−16]

Currently, there is a high level of interest in supercritical fluid chromatography (SFC) as an alternative to HPLC for the analysis and purification of compound libraries. In contrast to reversed-phase HPLC, SFC is a normal phase chromatography technique using a compressible fluid as the mobile phase. Typically, carbon dioxide (CO_2) is used as one component of the mobile phase, and methanol or methanol with an organic modifier is used as the other component. Supercritical fluids have lower intermolecular forces than those in normal liquids, resulting in lower viscosities and higher diffusion rates for the mobile phase used in SFC compared to HPLC.[17] SFC systems can therefore be run at higher flow rates than an equivalent HPLC system without excessive column backpressure or loss of resolution, leading to shorter run times per sample. This throughput advantage has been reported in the use of analytical SFC and SFC/MS for the analysis of combinatorial libraries.[18−20] Preparative SFC has the further advantage over preparative HPLC of producing purified fractions in a solvent that can be more readily removed. Evaporation of CO_2 occurs upon fraction collection, leaving purified compounds as solutions in methanol, which can be dried down much more rapidly than the aqueous fractions resulting from reversed phase preparative HPLC. In addition, SFC produces significantly lower amounts of hazardous waste solvent for disposal than HPLC, which becomes a significant advantage as compound numbers increase. Several groups have reported the use of preparative SFC for the purification of compound libraries,[21−25] with systems ranging from those using simple UV-triggered fractionation to mass-directed fraction collection and parallel sample processing.

At Abbott Laboratories, we have established a High-Throughput Organic Synthesis (HTOS) group to produce compound libraries by parallel synthesis as a service to drug-discovery chemists, using standardized reactions and monomer sets from core compounds supplied by the chemist.[26] A High-Throughput Purification (HTP) group provides purification support for HTOS as well as to medicinal chemists. For the majority of HTP samples, systems using preparative HPLC with UV or evaporative light-scattering detection (ELSD)-triggered fraction collection and subsequent flow injection analysis by mass spectrometry of selected fractions have been the tool of choice for >5 years.[27] These older systems are now being supplemented by systems based upon mass-triggered preparative HPLC and preparative SFC.[24,25]

The results of a recent study into the stability of compounds vs their TFA adducts in a repository compound

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Table 1. Summary of Library Purification Results

		preparative HPLC		preparative SFC	
library no.	samples purified	success	yield (%)	success	yield (%)
1	45	42	18	44	34
2	48	45	49	45	38
3	48	43	44	37	39
4	48	48	56	48	64
5	45	44	31	44	45
6	48	41	22	44	26
7	44	40	25	40	27
8	47	38	30	43	32
9	47	42	21	45	13
10	48	31	29	29	27
11	48	48	30	45	25
12	36	36	63	35	53
13	48	44	43	41	34
14	48	46	24	9	
15	48	45		46	
16	48	44	24	18	

collection[28] prompted us to purify a large number of compounds by both preparative SFC and reversed-phase HPLC. A series of libraries were synthesized using diverse cores and sets of monomers and were purified by both preparative HPLC (to give TFA adducts) and SFC (to give salt-free compounds). To the best of our knowledge, direct comparison of HPLC vs SFC purification for a significant number of compounds has not been previously reported in the literature. The results of the purification of these libraries using these two methods and implications for the selection of a purification technique are reported herein.

Experimental Section

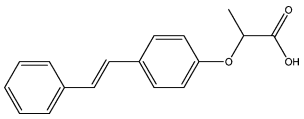
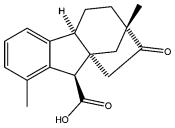
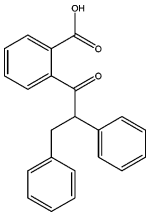
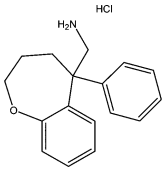
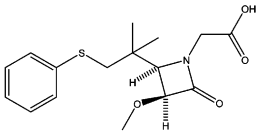
Analytical LC/MS was performed on a Waters ZMD mass spectrometer and Alliance HPLC system running under MassLynx 3.4 and Openlynx 3.4 software. The ZMD mass spectrometer was operated under positive APCI ionization conditions. The HPLC system comprised a Waters 2795 autosampler sampling from 96-well plates, a Waters 996 diode-array detector, and a Sedere Sedex-75 evaporative light-scattering detector (ELSD). The column used was a Phenomenex Luna Combi-HTS C8(2), 5 μm, 2.1 $\times$ 30 mm. A gradient of 10−100% acetonitrile (A)/0.1% trifluoroacetic acid in water (B) was used at a flow rate of 1.5 mL/min (0−0.1 min 10% A, 0.1−3.1 min 10−100% A, 3.1−3.9 min 100−10% A, 3.9−4.0 min 100−10% A).

SFC purification was carried out using a modified Berger Instruments PrepSFC system.[24] A manual version of the Berger system was integrated with a Gilson 232 autosampler for sample injection and a Cavro MiniPrep pipettor customized for fraction collection at atmospheric pressure.[24] Custom-designed collection shoes allowed collection into 18 $\times$ 150-mm tubes, and a methanol wash system allowed washing of shoes between fractions to maximize recovery and avoid cross-contamination of fractions. The column used was a Berger Instruments Diol, 60 Å, 6 μm, 21.2 $\times$ 150 mm. A gradient of 5−60% methanol with 10 mM triethylamine (A) and carbon dioxide (B) was used at a flow rate of 40 mL/min (0.0−0.5 min 5% A, 0.5−6.0 min 5−60% A, 6.0−7.5 min 60% A, 7.5−8.0 min 60−5% A). Samples were injected as solutions in 1.0 mL MeOH. Fractions were collected based upon UV signal threshold, and selected fractions were subsequently analyzed by flow injection analysis mass spectrometry using positive APCI ionization on a Finnigan LCQ using 70:30 MeOH/10 mM NH_4OH(aq) at a flow rate of 0.8 mL/min.

HPLC purification was performed on an Agilent 1100 series purification system which consisted of the following modules: Agilent 1100 series LC/MSD SL mass spectrometer with API-electrospray source; two Agilent 1100 series preparative pumps; Agilent 1100 series isocratic pump; Agilent 1100 series diode array detector with preparative (0.3 mm) flow cell; Agilent 35900E multichannel interface; LC-Packings Acurate 1:1000 flow-splitter; Gilson 215 liquid-handler with 819 injector fitted with a 2-mL loop; and Sedere Sedex-55 evaporative light-scattering detector (ELSD). The column used was a Phenomenex Luna Combi-HTS C8(2), 5 μm, 21.2 $\times$ 50 mm. A gradient of 10−90% methanol with 0.1% TFA (A) and water with 0.1% TFA (B) was used at a flow rate of 40 mL/min (0.0−0.5 min 10% A, 0.5−6.0 min 10−100% A, 6.0−7.0 min 100% A, 7.0−7.1 min 100−10% A, 7.1−8.0 min 10% A). The makeup pump for the mass spectrometer used 3:1 methanol/water with 0.1% formic acid at a flow rate of 1.0 mL/min. A tee downstream of the splitter divided the flow between ELSD and MS detectors with 0.45 mL/min flow to the MS. Library samples were injected in 1.0 mL 1:1 MeOH/DMSO. Fraction collection was triggered when the extracted ion chromatogram (EIC) for the target mass exceeded the threshold specified in the method.

All custom software was written in-house using Microsoft Visual Basic 6.0. The preparative SFC system was controlled using SFC ProNTo software (version 1.5.305.15) and custom software for autosampler and fraction collector control. Data were exported to custom software for fraction selection, MS analysis of fractions, and selection of fractions for drying down and archiving. Loop-injection mass spectra were acquired using a Finnigan LCQ running LCQ Navigator software (version 1.2) and a Gilson 215 liquid handler for fraction injection under control of custom software. Preparative HPLC system control was through Agilent Chemstation (Rev A.08.04) and Integ CC-Mode (Rev A.03.02) software. The data were exported through the use of Chemstation

Table 2. Core and Monomer Types for Libraries 1−5

no.	core	reaction type	monomers
1		acylation	2° amines
2		acylation	1° amines
3		acylation	1° amines
4		acylation	aliphatic acids
5		acylation	2° amines

macros to a custom Visual Basic data browser for review of MS data for each fraction and selection of fractions for drying down and archiving. Chemstation Macros were developed in-house using MacroPad 2.12 (Agilent Technologies). The custom data browser software used graphing controls from Measurement Studio 6.0 (National Instruments).

Results and Discussion

A total of 16 libraries, consisting of 48 samples per library, were used in this study. Each library member was synthesized on a scale of 16−21 mg. Libraries were evaluated by analytical LC/MS, and samples in which the expected product was confirmed were then submitted for purification. Samples were split in half and purified by both preparative HPLC and preparative SFC techniques. HPLC purification was mass-directed, with fraction collection triggered when the extracted ion chromatogram (EIC) for the target mass exceeded a specified threshold. SFC purification used the UV signal to trigger fraction collection, and fractions were subsequently analyzed by flow-injection analysis mass spectrometry to confirm the fraction containing the expected product. After removal of solvent from purified fractions, analytical LC/MS and NMR were used to confirm the purity and identity of each sample prior to weighing in order to calculate the yield. For each library, the number of samples in which the technique was successful, that is, succeeded in isolating the desired product, and the average yield for purified products are summarized in Table 1.

In general, both HPLC and SFC were successful in obtaining purified products. The average purified yield for the 16 libraries was virtually identical for both techniques: 34% for HPLC and 35% for SFC. These results were encouraging, considering the different approaches to fraction triggering and signal threshold used.

Several libraries were more successfully purified by HPLC than by SFC. Library **14** was an example in which the reactions did not go to completion, and the desired product was only a minor component of the crude mixture. The product was more successfully isolated by HPLC than by SFC; however, this was due largely to the advantages of mass-directed fraction collection, as compared to UV triggered fractionation. Despite the use of a low UV signal threshold for the SFC purification, the minor peak was often not collected. Using a low EIC threshold for the mass-triggered HPLC system was successful in isolating the product with the desired molecular weight, even when present in small amounts.

Reliability was the biggest difference between the two techniques. Despite recent advances in reliability of the SFC system, robustness was still a major concern. Problems included loss of communication between the instrument and software, pressure regulator failures, and occasional fraction collector problems due to jammed tubes. Although these were rarely occurring problems, the interruptions to automated purification strongly influence the practical sample throughput of the system. In particular, overnight running of libraries on the SFC system was limited. By contrast, overnight operation of the preparative HPLC system was trouble-free. Occasional failure to collect the product of interest was experienced due to user error in specifying a threshold for the EIC of the desired mass in the instrument method, but instrument problems were not experienced.

The 16 libraries chosen for this study were designed to contain a diverse set of core and monomer structures.

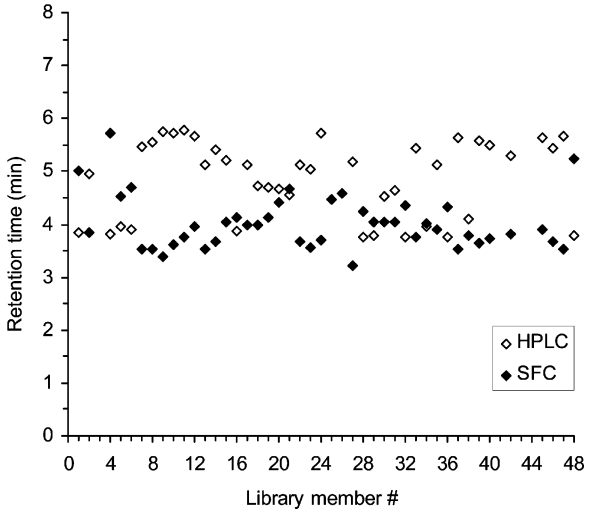


Figure 1. HPLC and SFC retention times for library **1**.

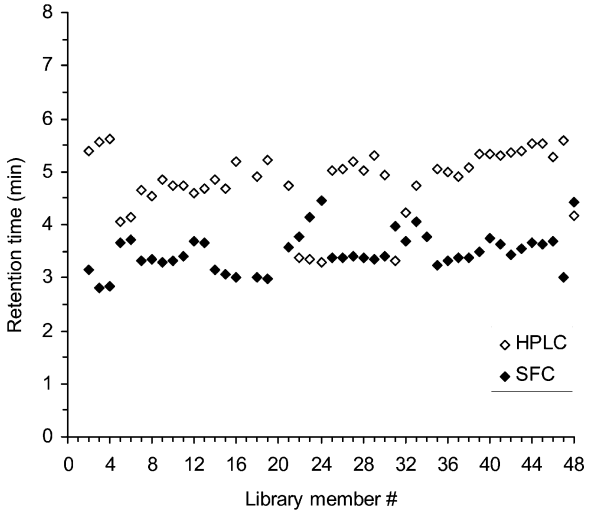


Figure 2. HPLC and SFC retention times for library **2**.

Standard diversification procedures were employed for the selection of cores such as to include the widest feasible range of structural types. Additionally, monomers were selected from our "diversity racks", which are designed to instill yet more structural diversity on a specific core molecule, and attached via variant reactions. The compounds in this study therefore represent a widely diverse set of structural types. Libraries **1**−**5** are listed in Table 2, and the purification results for these libraries were studied in greater detail. The retention times of the product peak for both techniques showed a fairly narrow distribution range for the 48 members of a library (Figures 1−5). This was especially evident in the preparative SFC case. Due to the wide range of structural types for compounds submitted to the high-throughput purification group, the generic methods we have developed are conservative with respect to throughput. In particular, the SFC methods have an extended dwell time at the end of the gradient to ensure any strongly retained material is eluted from the column. Even within this narrow time range, the peaks obtained by preparative SFC were well resolved.

Preparative SFC fraction collection has inherent difficulties due to the high flow of gaseous CO_2 that results when the pressurized solvent flow reaches atmospheric pressure at the point of collection. To avoid aerosolization of the compound-containing methanol stream, an efficient fraction collection scheme must be used. Failure to do this will result in lower product yields due to poor recovery of the collected fraction. Our modified fraction collection system, using custom collection "shoes" at atmospheric pressure, has been shown

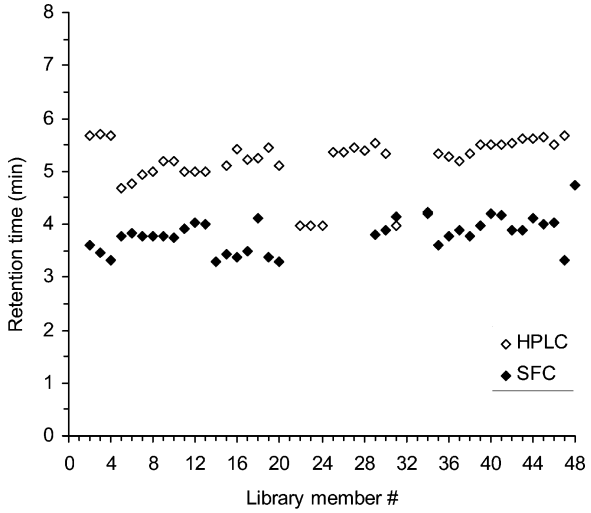


Figure 3. HPLC and SFC retention times for library **3**.

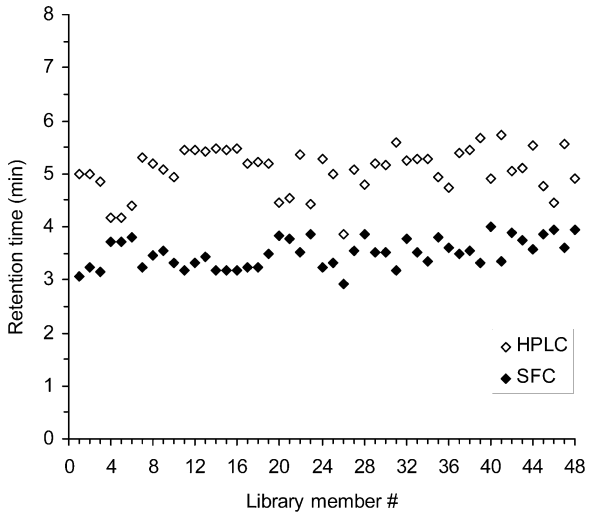


Figure 4. HPLC and SFC retention times for library **4**.

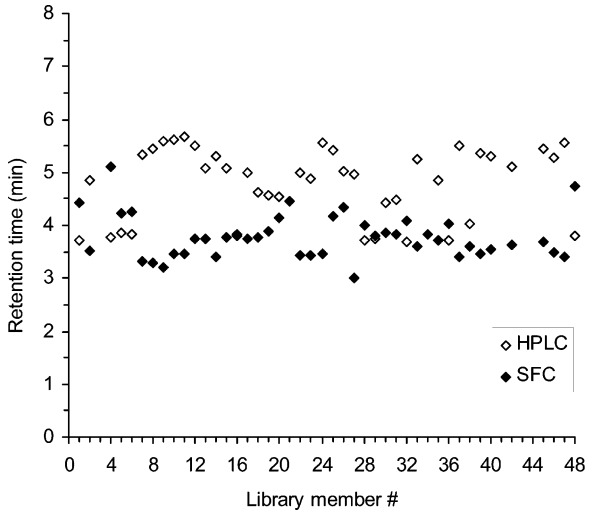


Figure 5. HPLC and SFC retention times for library **5**.

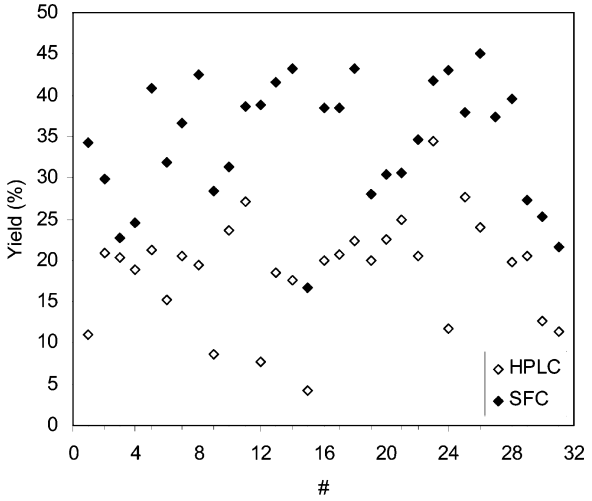


Figure 6. Purified product yields for library **1**.

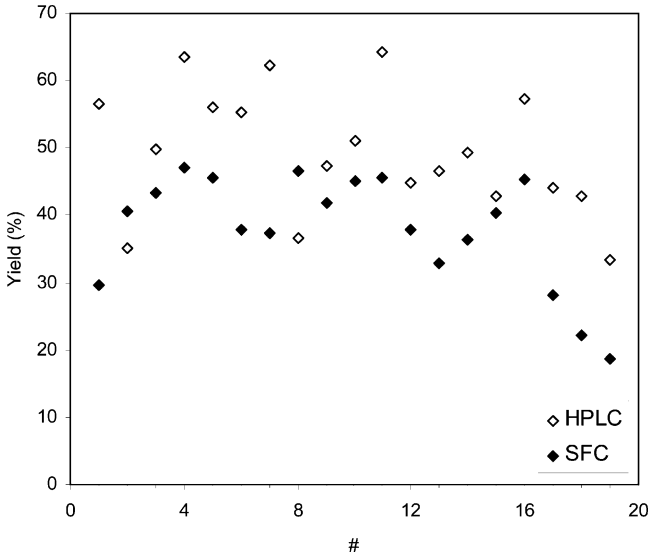


Figure 7. Purified product yields for library **2**.

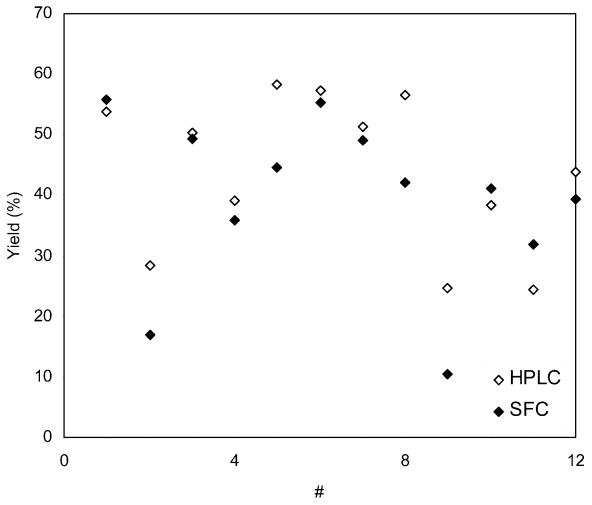


Figure 8. Purified product yields for library **3**.

to collect fractions with comparable efficiency to HPLC and give up to 98% recovery.[24] Nevertheless, a comparison of recovery for the two techniques over a wider range of compounds was of interest. Analysis of the purified yield for each library member for libraries **1**−**5** is summarized in

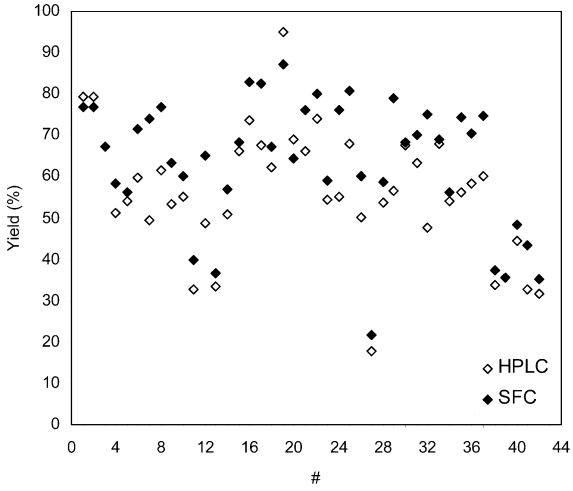


Figure 9. Purified product yields for library **4**.

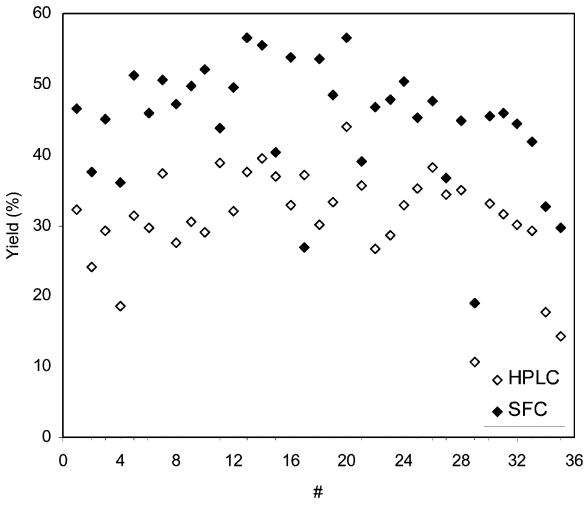


Figure 10. Purified product yields for library **5**.

Figures 6−10. In many cases, SFC purification gave a higher purified yield than purification of the same library sample by HPLC.

Conclusions

This study has provided an evaluation of the relative value for the purification of HTOS libraries over a wide range of structural types for each of HPLC and SFC. Although we have found that no single technique offers a clear advantage chromatographically, both were found to be acceptable for the general purification of diverse structural types.

Upon the basis of the results of this and other studies, we have made the decision at Abbott Laboratories to increase our capacity for SFC purification due to other advantages inherent in the technology, specifically, the ease with which solvent can be evaporated from fractions and the ability to provide products in salt-free form. The latter advantage may be important for compound storage when TFA salts are undesirable or for biological screening of purified compound when TFA can cause problems with the assay.

Clearly, the largest potential advances in the area of purification of parallel synthesis libraries would be a combination of MS and SFC, offering the advantages as were seen by mass-triggered HPLC in this study, with the practical advantages of SFC. Although custom SFC/MS systems have

been developed to accomplish this,[22,23] it remains to be seen whether the technology will gain general acceptance and be provided as a single vendor system.

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