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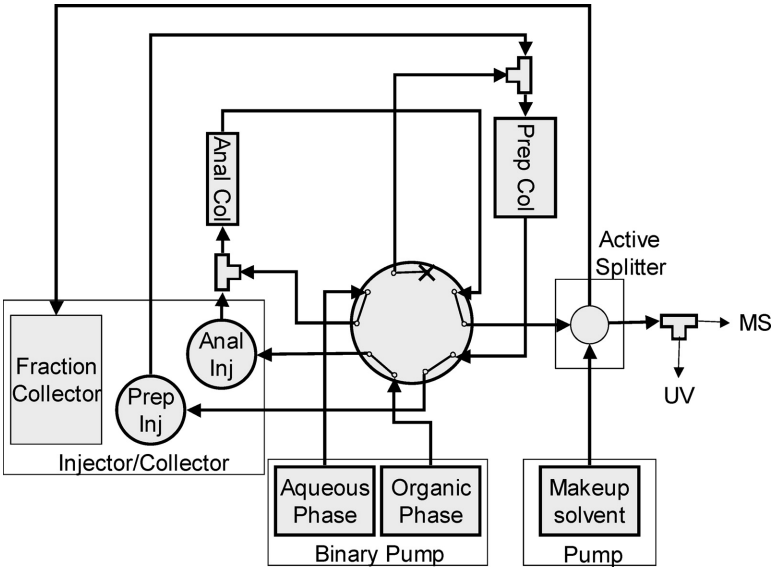

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Preparative LC−MS Purification: Improved Compound-Specific Method Optimization

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One of the remaining challenges in providing effective preparative LC−MS purification is balancing throughput and compound purity. We describe here an approach to optimizing preparative LC−MS methods that provides significantly better chromatographic resolution and, hence, better compound purity than generic preparative LC methods consuming the same amount of time. This approach is easier to implement, is more rugged, and permits significantly greater flexibility than previously reported approaches. The instrument configurations and protocols presented here are specifically tailored for open access support, but the basic approach is equally suitable and effective in high-throughput situations.

Introduction

Preparative liquid chromatography−mass spectrometry (prep LC−MS) is an effective and highly efficient approach to compound purification that is rapidly becoming ubiquitous in discovery chemistry programs.[1−8] Highly advanced and reliable systems are available from several instrument vendors. Methodologies and protocols to fully exploit the advantages of these systems are, however, in the early stages of development.

The conundrum in developing effective protocols for preparative LC−MS purification is balancing throughput and compound purity. The conventional practice of iterative scale-up and method refinement to optimize chromatography[9] in order to achieve required purity can be laborious and time-consuming, negating the potential efficiency of prep LC−MS and making poor utilization of an expensive and valuable resource. Conversely, employing fast generic gradient methods to achieve throughput does not always produce the needed compound purity.

We previously reported a simple but effective approach of "compound-specific method optimization" for prep LC−MS that employs a preestablished set of "focused" prep LC methods, each focusing on a fraction of the accessible hydrophobic range.[4] The crude sample is subjected to a fast analytical scale or "preprep" LC−MS analysis, and the retention time (RT) of the product is used to select the most appropriate of the focused prep LC methods for the purification. Similarly, the MS response from the preprep analysis is used to select an appropriate MS fraction method from a preestablished set of fractionation methods. The principal shortcomings of this discrete method selection approach are (1) it is difficult to create and effectively validate the set of focused prep LC methods and (2) the results can be less than optimal for compounds with preprep RTs near the boundary between contiguous focused prep LC methods.

We report here a simple variation on that strategy that eliminates the drawbacks of the earlier approach and permits significantly greater flexibility in the optimization procedure. In this approach, the relationship between the preprep LC−MS retention time and the mobile phase composition at which elution occurs in the prep LC gradient (the scale-up relationship) is established for the preprep LC−MS/prep LC−MS system from an analysis of standards. Prior to purification, the crude sample is subjected to the preprep LC−MS analysis, and the mobile phase composition at which the product will elute in prep LC run is estimated from the observed preprep RT and the known scale-up relationship. A prep LC method is then created with a shallow gradient focused around this elution composition. The length and slope of the gradient may be chosen to fit the specific needs of the separation. An optimized fraction method setting the threshold for fractionation is created from the observed preprep MS response in an analogous manner.

This prep LC optimization protocol is fundamentally the same as the recently reported accelerated retention window (ARW) method;[10] however, ARW uses a nonspecific detector (UV or ELSD) to trigger fractionation, and product targeting is accomplished by estimating the chromatographic "retention window" with great precision. Even with a relatively narrow target retention window, multiple components may be collected, requiring subsequent deconvolution by LC−MS analysis. In addition, if structure-dependent variations in the scale-up relationship result in the product's elution outside the target retention window, the product is lost. The ARW approach is well-suited and highly effective for high-throughput purification of analogous products in an "expert operator" environment, but may not be appropriate for chemistry programs exploring highly diverse chemical space or operation in an open access environment.

The complexities and shortcomings of this approach to prep LC method optimization are greatly reduced when mass-directed fractionation is employed. The higher specificity of mass-directed fractionation reduces the occurrences of

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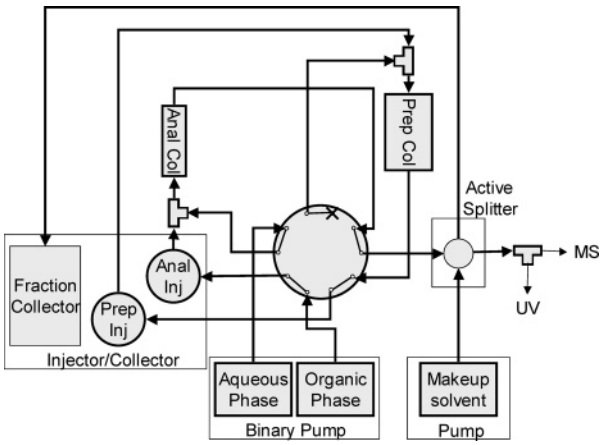

Figure 1. Schematic view of preprep LC−MS/prep LC−MS instrument configuration.

equivocal fractions and allows the product fraction to be collected even if the product retention time deviates drastically from the expected value. This enables the utilization of strategies that enhance the overall process even though they may compromise the precision of the scale-up calculation, such as fast-gradient LC for the preprep analysis; more highly focused prep LC gradients (methods using shorter, shallower gradients); simplified validation protocols; and ignoring structure-dependent variations in the scale-up calculation. The practical advantages realized by combining compound-specific method optimization with mass-directed fractionation are higher compound purity, shorter total purification time, simplified implementation, and more robust purification protocols.

As with the previously reported method, a prime consideration in developing the approach presented here was simplicity of implementation. The optimization protocols are accomplished without alteration of the vendor provided hardware or software. The combined preprep LC−MS/prep LC−MS configuration is created by a relatively simple reconfiguration of the plumbing of the standard hardware. The method optimization software is called from the vendor's instrument control software but operates separate from the vendor software. No modification of the vendor software is needed to implement the optimization protocols.

The instrument configuration, protocols, and software presented here were developed for use within an "open access" environment. Although we will describe a relatively sophisticated graphical user interface intended to make the purification process easily accessible to casual users, this is not necessary for the implementation of this approach. With minor modifications, this basic approach is also well-suited for high-throughput and "expert" applications of preparative LC−MS.

Experimental Section

The open access preprep LC−MS/prep LC−MS configuration is represented schematically in Figure 1. The binary pump is the Waters 2525, and the makeup pump is the Waters Reagent Manager. The injector/fraction collector is the Waters 2676, the 10-port switching valve is part of the Waters Column/Fluidics Organizer, and the splitter is the Waters Active Flow Splitter. The mass spectrometer is the Waters ZQ2000, and the UV detector is the Waters 2996 photodiode array detector. The mass spectrometer, LC, and mass-directed fraction collection are controlled via Masslynx version 4.0 with Fractionlynx.

The "at-column dilution" mixers[11,12] are the Upchurch static mixing tee, part number U-466. All tubing between the LC pumps and the injector valves is 0.030-in.-i.d. stainless steel, the tubing from the analytical injector to the column is 0.010-in.-i.d. stainless steel, and from the preparative injector to the prep column is 0.030-in.-i.d. stainless steel. All tubing after the columns is PEEK tubing of an appropriate size. The preprep column is a Waters SymmetryShield C18, 4.6 × 50 mm, 5-μm particle size with an Agilent 4.6 × 12.5 mm Zorbax SB-C18 guard column. Static mixing tees cannot be relied upon to provide adequate mixing of the mobile phase at flow rates less than ∼10 mL/m; thus, a guard column is used in the preprep channel to improve mixing and ensure good chromatographic peak shape.[11] The preparative column is a Waters SymmetryShield C18, 19 × 100 mm, 5-μm particle size. No guard column is utilized on the preparative channel. This is because effluent distribution is not well-controlled in most guard columns, and flow through the frit is often uneven. Hydrophobic compounds that may precipitate from solution at the initial gradient conditions can accumulate on the guard column frit in the regions of poor flow. At the large sample loadings possible with at-column dilution, the rise in back pressure can become problematic. Conversely, effluent distribution onto most preparative columns is adequate so that this problem does not occur. Both preparative and preprep LC columns are flushed daily with at least 10 column volumes of methanol/0.1% TFA then acetonitrile/0.1% TFA. Preparative column lifetime is typically greater than 3000 injections (often much greater).

The organic phase is acetonitrile with 0.1% TFA (v:v), the aqueous phase is water with 0.1% TFA, and the make up solvent is methanol with 0.01% TFA. The preprep LC method and all prep LC methods are summarized in Table 1. All methods are started at a low flow rate and ramped up to the full flow rate over 15−30 s to minimize pressure spikes during the injection/sample loading cycle. Flow is turned off at the end of each run to eliminate the potential for pressure spikes when switching between the preparative and preprep channels. All of the samples used in this work were dissolved in methanol, acetonitrile, DMSO, or a combination thereof in various proportions as required to attain adequate solubility. The injection volume for preprep LC−MS analyses was 40 μL and for the prep LC−MS runs was 2.0 mL. For a listing of all standard compounds used in this work, see ref 4.

Results

Scale-Up Relationships. Table 1 summarizes the preprep and prep LC methods discussed in this section. Figure 2 shows the relationship between preprep retention time and the generic prep method elution composition (calculated mobile phase composition at the "top-of-the-peak" retention time, corrected for column dead volume) for 16 diverse standard compounds. (For a full listing all standard com-

Table 1

preprep			generic prep			"standard" focused prep			"long" focused prep		
time	flow	%AcN	time	flow	%AcN	time	flow	%AcN	time	flow	%AcN
0	2	10	0	5	10	0	5	initial	0	5	initial
0.25	5	10	0.5	30	10	0.5	30	initial	0.5	30	initial
4.25	5	100	1.0	30	10	1.0	30	initial	1.0	30	initial
4.5	5	100	6.0	30	100	6.0	30	final	11.0	30	final
4.6	5	10	7.0	30	100	6.1	30	100	11.1	30	100
4.9	5	10	7.25	30	10	7.0	30	100	12	30	100
5	0	10	7.75	30	10	7.25	30	10	12.25	30	10
			8	0	10	7.75	30	10	12.75	30	10
						8.0	0	10	13	30	10

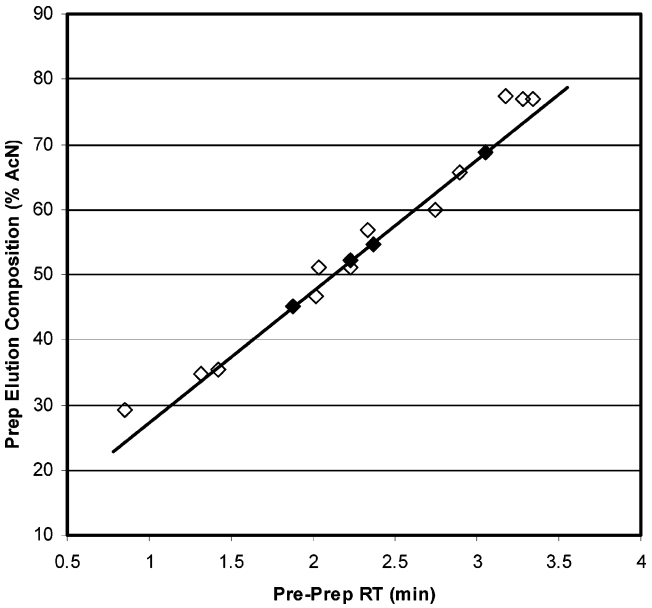

Figure 2. Scale-up relationship: preprep retention time vs calculated prep elution composition. Solid data points represent the validation standards: tetracaine, dibucaine, reserpine, and clofazamine.

pounds, see ref 4.) The relationship is well-approximated by a linear fit. Analogous data from many other commercial and in-house standards correlate well with the linear relationship shown in Figure 2. To a first approximation, this relationship appears to be characteristic of the specific preprep LC−MS/prep LC−MS system and to be applicable to a wide range of chemical structures. The deviations from the linear fit are reproducible and are the result of minor structure-dependent deviations in the scale-up relationship. No systematic effort to predict and adjust for structure-dependent deviations has yet been attempted; however, this would be an obvious next step in the development of this method optimization protocol.

Elution compositions calculated from generic prep LC and focused prep LC retention times of the same compound always differ somewhat. For example, for clofazamine the elution composition calculated from the retention time obtained with the generic prep LC method is 68.7% acetonitrile; with a focused method employing a 20% gradient, the apparent elution composition is 57.1% acetonitrile. This deviation is important because generic prep method retention times are used to determine the scale-up parameters for the focused prep methods used in purification, see below. The deviation is, obviously, at least partly the result of not accounting for the entire dead volume of the preparative flow path (injector loop, transfer lines, etc); however, attempts to precisely account for all dead volume failed to completely eliminate the difference. The difference appears to be independent of compound structure, essentially the same for all focused preparative LC methods reported here, characteristic of each individual LC−MS system, and constant over the long term. Consequently, we chose to treat this deviation empirically, defining it as an instrument constant and estimating its value for each system through iterative approximation.

Four standards that correlate well with the linear fit for the full set of standards were selected for establishing the scale-up relationship of new systems and routine system validation. These are tetracaine, dibucaine, reserpine, and clofazamine, indicated by the solid data points in Figure 2. Estimating the scale-up relationship for a system then consists of analyzing a mixture of these four standards (at any convenient concentration) with the preprep and generic prep LC−MS methods and determining the slope and intercept of a linear fit to a plot of preprep RT vs preparative elution composition. The focused gradient prep LC elution composition for any compound is then estimated from its preprep RT according to

$$C = m\mathrm{RT} + b + \Delta$$

where C is the focused gradient prep elution composition (% acetonitrile), m and b are the slope and intercept, respectively, of the linear fit to the scale-up relationship (preprep RT vs prep elution composition for the standard mixture), and Δ is the "instrument constant" described above. The value of Δ is determined by iteratively adjusting its value, estimating the elution composition of an appropriate standard, and running the standard using the prep LC gradient focused about this composition until the standard elutes at the target retention time. Once established, the value of this parameter appears to be stable unless changes are made to the configuration of the preparative channel.

Figure 3 shows the preprep LC−MS data and focused prep LC−MS data for two standard compounds in simple mixtures (~10 mg each component). In both examples, the focused prep LC gradient is 5 min long, the gradient slope is 4% increase in acetonitrile composition per minute (4%/min), and the estimated elution composition occurs 3 min into the gradient. With the 1-min sample loading phase at the start of the focused prep LC method, the expected retention time is 4 min. The gradient specifics are noted in Figure 3. In the

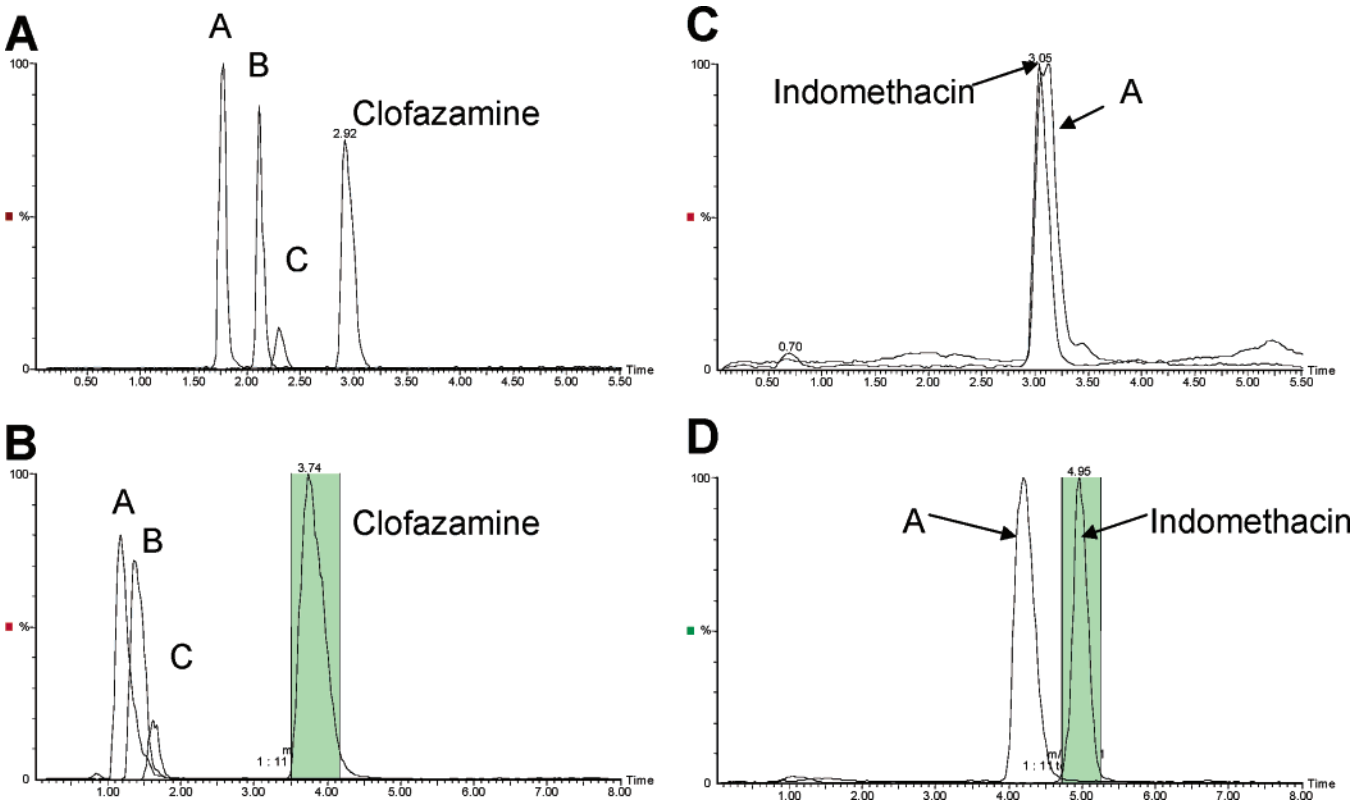

Figure 3. (A) Preprep LC−MS data for clofazamine mixture. (B) Focused prep LC−MS data for clofazamine mixture; gradient from 41.3 to 61.3% acetonitrile. (C) Preprep LC−MS data for indomethacin mixture. (D) Focused prep LC−MS data for indomethacin mixture; gradient from 44.0 to 64.0% acetonitrile.

first example, clofazamine in Figure 3A and B, the scale-up procedure gives approximately the expected result, with focused prep elution occurring at 3.74 min. The elution order of the components is the same in the preprep LC−MS analysis and the prep LC−MS run. In contrast, the standard indomethacin, Figure 3C and D, elutes at 4.95 min into the focused prep run, significantly later than expected. Furthermore, the elution order of the indomethacin and component A has reversed in the focused prep run. Indomethacin exhibits a considerable structure-dependent deviation in the scale-up relationship. Despite the deviation, however, the clofazamine does elute within the separating gradient and is collected by the mass-directed fractionation. In actual usage, with this degree of gradient focusing (20% change in acetonitrile composition in 5 min), virtually all compounds purified on this system have eluted within a viable range, with RTs between 3 and 5 min.

This system does not precisely match the flow rates and sample loadings employed in the preprep and preparative LC methods. These differences likely contribute to the magnitude of the structure dependent deviations observed in the scale-up calculations. However, system and method robustness were deemed more important in this application than optimization of the scale-up calculation. For example, to avoid consuming excessive amounts of sample in the preprep LC−MS analysis and to prevent clogging of the relatively narrow bore tubing used in the preprep channel, the preprep injection volume was set to 40 μL (∼2% of the crude sample). Additionally, to affect good mobile phase mixing, thereby ensuring acceptable LC−MS peak shape, the preprep flow rate was set to 5 mL/min. The 4.6-mm-i.d. column selected for the preprep analysis, then, represents a compromise between matching the linear velocity of the preparative flow (prep = 1.8 mm/s; preprep = 5.0 mm/s) and the preparative sample loading (prep = 70 μL/mL of column volume; preprep = 48 μL/mL of column volume). Despite these disparities, the precision of the scale-up calculation is quite satisfactory for the degree of gradient focusing utilized in the methods and applications presented here.

Controlling Prep LC Resolution. Once the elution composition for the product has been estimated from the preprep RT and scale-up relationship, the specifics of the focused gradient may be selected to give the required resolution, throughput, and method robustness. The resolution achieved with a generic or focused prep LC method (for a given column and flow rate) depends primarily on gradient slope (the rate of change in mobile phase composition) and is approximately independent of the gradient length. This can be demonstrated with a mixture of the standards cortisone, dibucaine, and reserpine (∼10 mg each in methanol) using various gradient lengths and slopes for generic and focused methods. The focused gradients were centered on the estimated elution composition for the dibucaine. The separation of the three compounds with the "standard" focused LC method is shown in Figure 4A, and the results for all methods are summarized in Table 2. Figure 4B shows the resolution obtained for the cortisone/dibucaine and dibucaine/reserpine pairs as a function of gradient slope. (Resolution is defined as the difference in peak retention times divided by the sum of the full width at half height for the peaks.) As expected, the chromatographic resolution

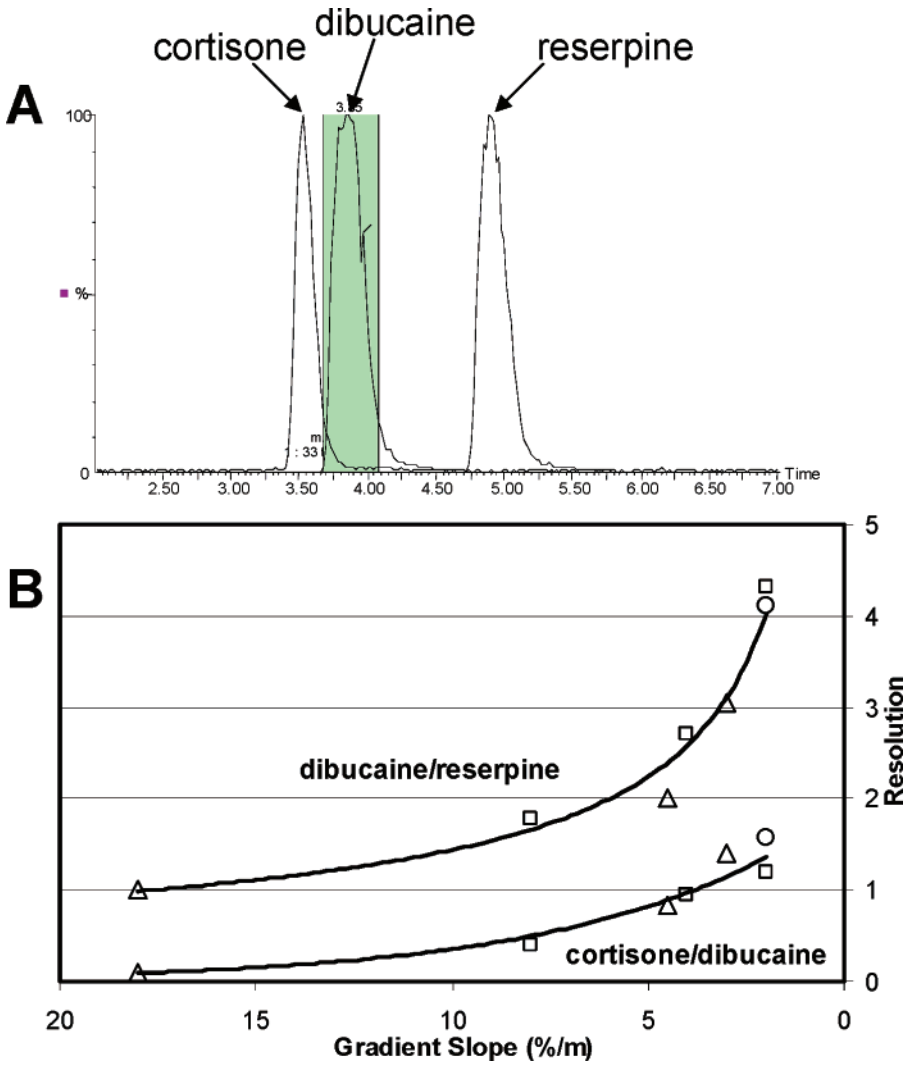

Figure 4. (A) Standard focused prep LC−MS separation for cortisone, dibucaine, reserpine mixture; gradient from 27.3 to 47.3% acetonitrile. (B) Prep LC−MS resolution as a function of gradient slope: (△) generic gradient, (□) 5-min focused gradient, (○) 10-min focused gradient. Resolution = (RT2 − RT1)/(fwhh1 + fwhh2).

Table 2

gradient type	slope (%/min)	length (min)	resolution (cortisone/dibucaine)	resolution (reserpine/dibucaine)
generic	18	5	0.05	1.0
generic	4.5	20	0.84	2.01
generic	3	30	1.40	3.04
focused	8	5	0.40	1.78
focused	4	5	0.94	2.70
focused	2	5	1.20	4.31
focused	2	10	1.58	4.11

increases with decreasing gradient slope. The resolution observed with the generic and focused methods exhibit approximately the same functional dependence on gradient slope.

Although resolution is approximately independent of the gradient length, minor differences in resolution are occasionally observed between focused prep LC methods utilizing the same gradient slope but different gradient lengths. This can be seen in Table 2 by comparing the 5- and 10-min focused gradients at 2%/min gradient slope. The resolution of the cortisone/dibucaine pair is ∼30% greater with the longer method, whereas the resolution of the dibucaine/reserpine pair decreases slightly with the longer gradient. These results are reproducible. The magnitude and direction of this effect appear to be compound-specific. These observations suggest that the magnitude of the structure-dependent deviations in the scale-up relationship may be dependent upon how long the product is retained on the column. Consider, for example, a product with a deviation in the scale-up relationship causing it to elute at a longer than expected retention time. The resulting shift in RT increases the product's separation from earlier eluting components while decreasing its separation from later eluting components. Now, if the magnitude of the deviation were to increase with increasing length of the gradient (increasing length of time the product is retained on the column) then

Table 3

compd	preprep max int[a]	calcd threshold[a,b]	prep max int[a]	threshold max int
indomethacin	0.488	0.162	1.73	9.4%
cortisone	1.32	0.436	3.11	14.0%
dibucaine	5.22	1.72	10.5	16.5%

[a] Basepeak intensity for target mass, $\times 10^6$. [b] Threshold = max preprep intensity/3.

the resolution would shift as observed for dibucaine in Table 2; resolution from earlier eluting peaks would increase while resolution from later eluting peaks would decrease. Further study of this phenomenon is needed.

Deviations in the scale-up relationship result in shifts in the eluting composition and, consequently, in the product eluting at a different retention time than intended. The shallower the slope of the gradient, the greater the resulting shift in product retention time. At a gradient slope of 2%/min, deviations of more than 2 min from the intended product RT have been observed. If a short gradient (e.g., 5 min gradient) were being employed, deviations of this magnitude would lead to the product's eluting near the void volume or the column rinse phase of the prep LC method. While the mass-directed fractionation would still collect the product under these conditions, the integrity of the separation and, hence, the purity of the product could be compromised. Consequently, our "standard" focused prep LC method utilizes a 5-min gradient with a slope of 4%/min. This combination yields a rugged preparative LC method that provides both acceptable chromatographic resolution (approximately equivalent to a 20−25-min generic gradient) and acceptable throughput (total time of preprep LC−MS analysis and prep LC−MS purification is ∼15 min). If additional resolution is required, users may select to use a "long" method employing a 10-min gradient with a slope of 2%/m.

Threshold for Fractionation. Previously, we reported that the relationship between mass spectrometric responses observed under preprep LC−MS and prep LC−MS conditions exhibited a strong dependence on product structure. This made estimating an appropriate threshold for prep LC−MS fractionation from the observed preprep LC−MS response somewhat complex. This dependence has been greatly reduced for the current system by increasing the split ratio (from 1:1000 to ∼1:40 000) and adding a low concentration of TFA (0.01% V:V) to the methanol makeup flow (see Figure 1). Under these conditions, an appropriate threshold for fractionation may be estimated as a simple fraction of the maximum intensity observed at the target mass in the preprep LC−MS analysis. This is demonstrated in Table 3 with three diverse compounds: an acidic compound (indomethacin), a neutral compound (cortisone), and a basic compound (dibucaine). The threshold for fractionation was set to 1/3 the maximum intensity for the target mass in the preprep LC−MS analysis. The "ideal" threshold for fractionation would be 10% of the maximum target mass

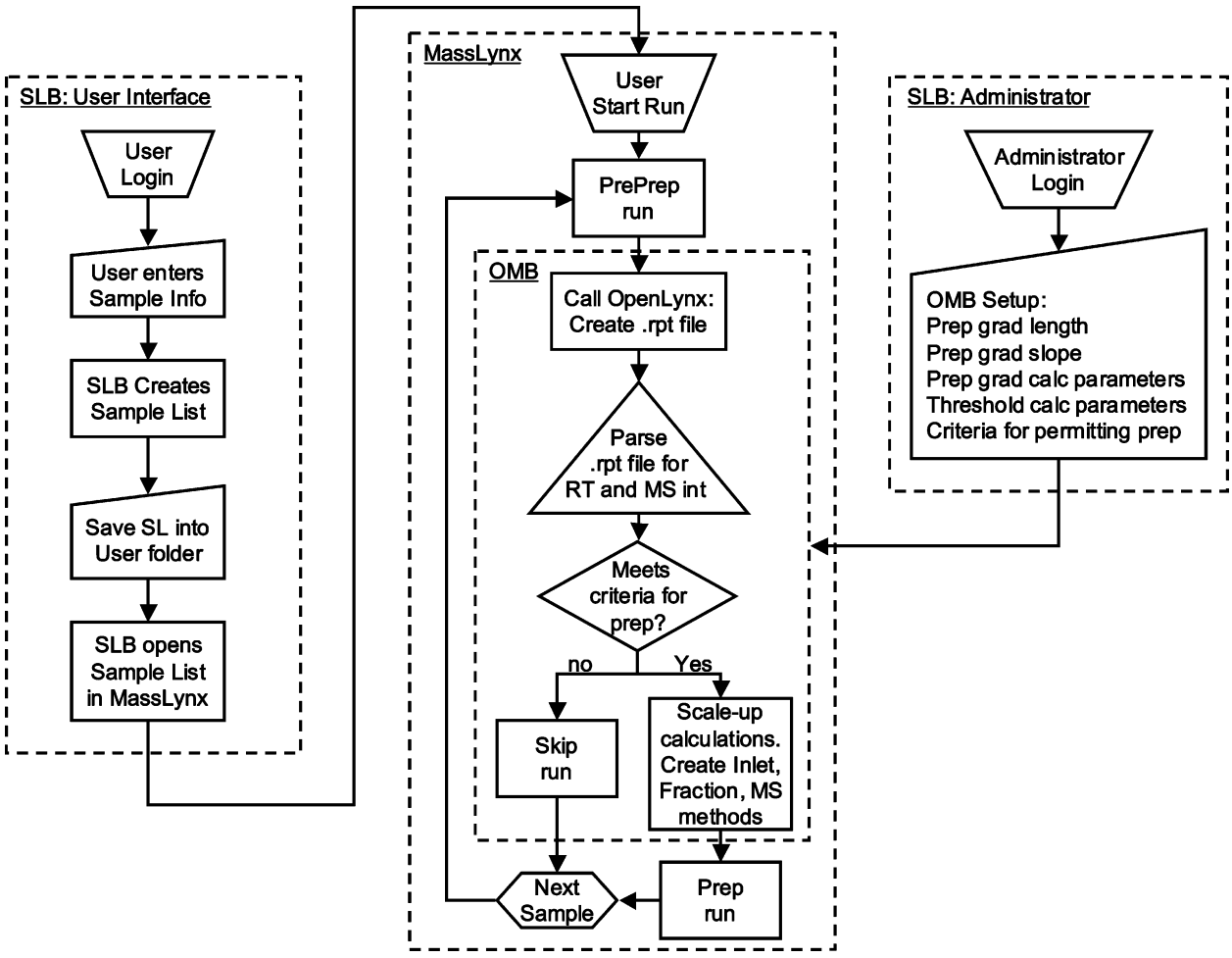

Figure 5. Flow diagram for Sample List Builder (SLB) and Optimized Method Builder (OMB) and their interaction with MassLynx.

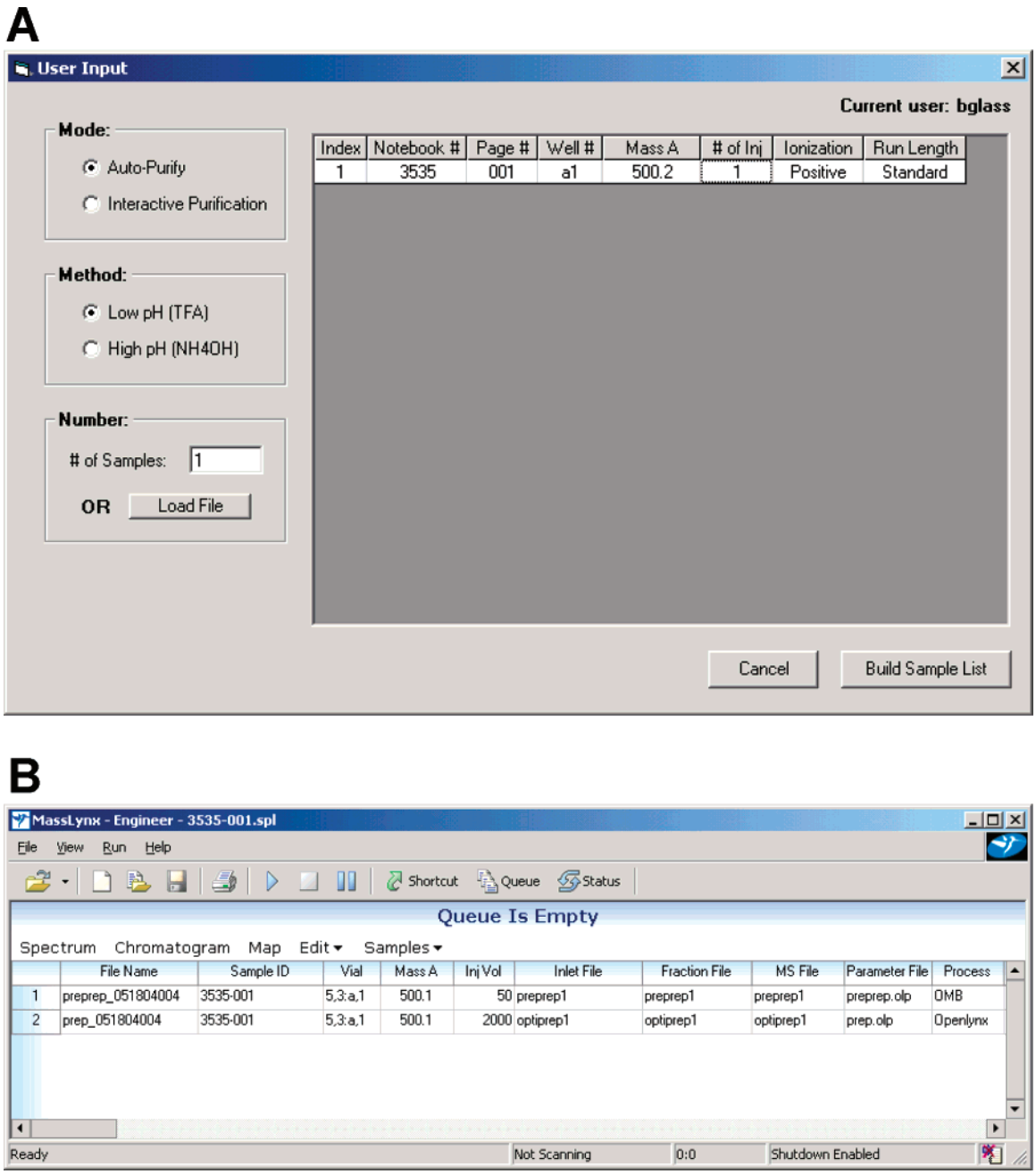

Figure 6. (A) Sample List Builder, sample information input page. (B) Sample List for single purification. Line 1 runs preprep LC−MS analysis and OMB; line 2 runs optimized prep LC−MS purification.

intensity in the prep LC−MS run; in practice an "acceptable" range is 5−20%. For all three compounds, the thresholds set from the preprep LC−MS analyses were well within the acceptable range.

Implementation. The prep LC−MS systems described here support a diverse chemistry program and operate in an open access environment. To address the diverse purification needs of the group, each prep LC−MS system provides at least two sets of prep chromatography conditions; all systems provide the SymmetryShield aqueous/acetonitrile/TFA conditions described in the Experimental Section and one or more alternate sets of conditions. To work within an open access environment, the optimized purification protocols must be simple, transparent, and rugged. To accomplish this, two programs were created using Microsoft Visual Basic 6.0 Sample List Builder (SLB): this program queries the user for sample information, creates the Sample List containing all information needed to perform the optimization and purification, loads the Sample List into MassLynx, and contains all administrative functions for the protocol. Optimized Method Builder (OMB): this program performs the scale-up calculations and creates the optimized methods (MS, LC, and fraction method files) for the preparative run. The operation of these programs and their interaction with MassLynx are summarized in Figure 5.

A typical SLB user input screen is shown in Figure 6A. The alternate conditions on this system utilize a Waters XTerra column and an aqueous/acetonitrile/ammonium hydroxide (0.1% v:v) gradient. The user begins by selecting a set of chromatographic conditions (either "low pH (TFA)" or "high pH (NH_4OH)") and whether to run OMB in automatic or interactive mode. The interactive mode pauses

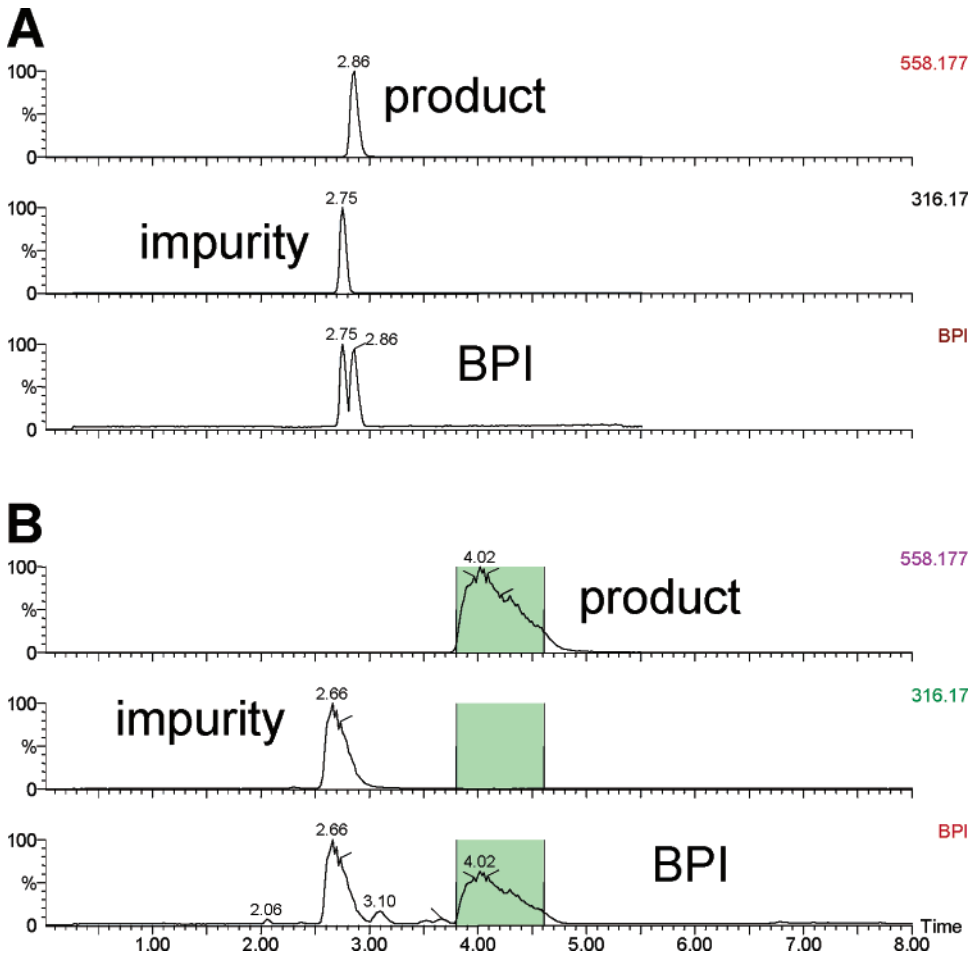

Figure 7. (A) Preprep LC−MS analysis for simple reaction mixture. (B) Focused prep LC−MS purification; 5-min gradient from 35.1 to 55.1%.

the Sample List after the preprep analysis, displays the preprep LC−MS data, and permits the user to select between the "standard" gradient, "long" gradient, or "skip" method for the prep LC−MS run. The automatic mode uses the preselected run length and administrator-specified criteria for determining whether to automatically proceed to the prep run or skip the run. The user specifies the number of different samples to be purified and either manually enters the required sample information into the grid or loads the information from an EXCEL spreadsheet. The user then selects "Build Sample List", and SLB creates the Sample List, queries the user for a name for the Sample List, saves the Sample List into the user's folder, and opens the Sample List in MassLynx. An example of a Sample List for the purification of a single sample is shown in Figure 6B. The first line of the Sample List executes the preprep LC−MS analysis and OMB program. The second line is the prep LC−MS run. The user initiates the Sample List run from MassLynx.

The OMB program is launched from the "process" field on the preprep line of the Sample List. The program uses OpenLynx to reduce the preprep LC−MS data according to the specified "parameter file". It then parses the OpenLynx report (.rpt file) for the product retention time and response; checks that the RT and response at the target mass meet the criteria established by the administrator for permitting purification to proceed; calculates the prep elution composition and threshold for fractionation; and creates and saves the MS file, Inlet file (prep LC method), and Fraction file. MassLynx then proceeds to the prep line of the Sample List and executes the optimized prep LC−MS run.

The success of the scale-up procedure is highly dependent on the condition of the prep and preprep columns and the overall system. Additionally, because these are open access systems, it is imperative that their performance and condition be monitored and maintained systematically and frequently. Consequently, we validate the scale-up computation and purification process on each system daily with the mixture of four standards described above. The criteria for passing validation are as follows: the target compound is collected (we generally collect the clofazamine because this is a highly colored compound and gives a visual indication that the fractionation timing is nominally correct), the fraction width is consistent with historical data, the retention time of the target compound with the focused prep LC method is near the expected value (∼4 min for the standard method), and the preprep LC−MS peak shape and retention times are consistent with historical data. The column maintenance protocol (described in the Experimental Section) and validation protocol require ∼30 min/day.

System Performance. There are numerous criteria that might be used to evaluate the performance of an open access purification operation. Three of the more important generic criteria are (1) the preparative chromatography provides needed compound purity (provides sufficient component

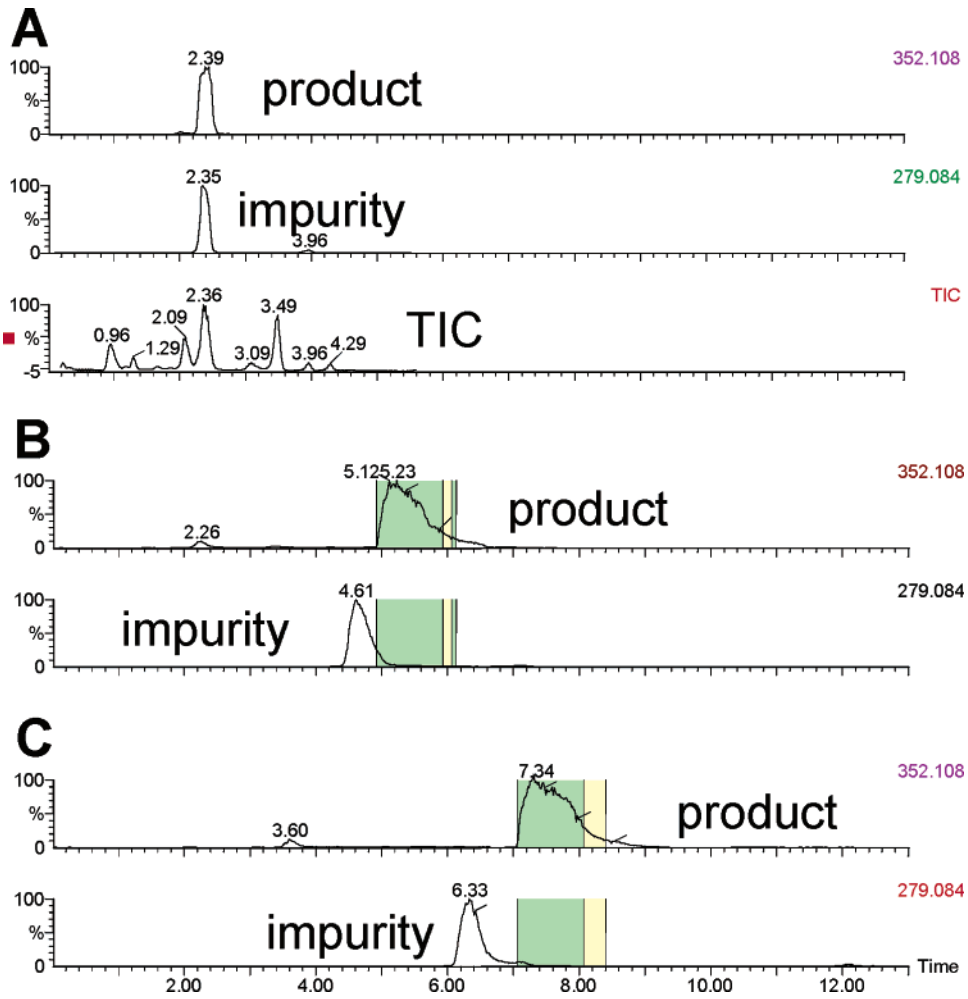

Figure 8. (A) Preprep LC−MS analysis of reaction mixture. (B) "Standard" focused prep LC−MS purification; 5-min gradient from 29.3 to 49.3%. (C) "Long" focused prep LC−MS purification; 10-min gradient from 29.3 to 49.3%.

separation) in a time short enough to meet sample throughput requirements, (2) the system provides adequate flexibility in meeting the specific resolution needs of each separation, and (3) the purification is very rugged (that is, an acceptable result is obtained in all cases for which appropriate chromatographic conditions were selected by the user, and in no case is the product lost). None of these criteria can be achieved absolutely or proved rigorously; however, we offer here a few examples to demonstrate that the system and approach described here do at least approach these goals.

Figure 7A shows the preprep LC−MS analysis of a reaction mixture containing the product, one major impurity, and several minor impurities. The product and major impurity are ∼80% resolved by the generic preprep LC gradient. The standard focused prep LC method (5-min gradient at 4%/min) separates the impurity from the product with nearly 1 min of baseline between the two (Figure 7B). In addition, several minor components that were hidden under the major peaks in the preprep analysis are completely separated from the product in the focused prep run. The total purification time (preprep analysis and prep run) was ∼15 min. From the analysis in Table 2, a comparable separation using a generic prep LC method would have required a 20−25-min gradient. The total purification time using a generic prep LC method (including injection time and column recovery time) would be ∼25−30 min. The increase in efficiency will be greater the higher chromatographic resolution needed to achieve adequate product purity. Efficiency will also be much greater when multiple prep LC−MS runs are required to purify a sufficient amount of product.

The preprep LC−MS analysis in Figure 8A reveals a major impurity that virtually coelutes with the product. The standard focused prep method removed ∼95% of the impurity from the product (Figure 8B), but the collected fraction did not meet the purity requirements for this compound. The "long" focused prep LC method (10-min gradient at 2%/min) improved resolution between product and impurity by ∼50% (Figure 8C) and resulted in adequately pure product (>98%). This approach to prep LC method optimization permits significant flexibility in creating methods to meet specific resolution needs. In principle, one could create methods with gradient slopes and lengths to precisely match the resolution requirements of the purification. In practice, however, it is difficult to fully automate such a procedure; it is reasonably effective and far easier to offer the user a selection of a small number of method options.

Figure 9 shows some of the results from an overnight purification of a small library of 48 compounds of diverse properties. This compound library exhibited many of the challenges common to purifying discovery chemistry compounds. Most of these compounds exhibited minor deviations in the scale-up relationship; most products eluted at retention

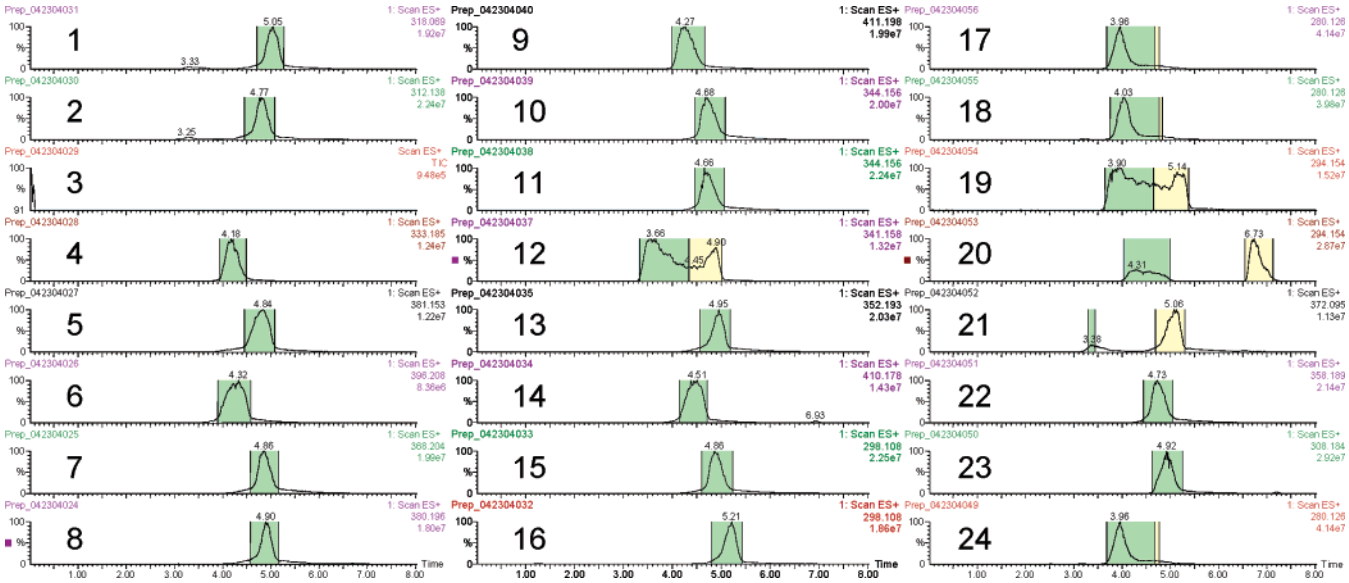

Figure 9. Standard focused prep LC−MS purifications for 24 of 48 reaction mixtures; target mass traces.

times longer than the target RT of 4.0 min. The average retention time was 4.61 min. A number of the components exhibit minor peak asymmetry; both fronting and tailing are observed. Two components exhibit significant peak distortion (traces 12 and 19), presumably due to interactions with the column stationary phase. One sample exhibited two peaks at the target mass (trace 20). For one sample, the chemist input the wrong mass for the product (trace 3). In all, 47 of the 48 compounds were successfully purified in the overnight run. The single failure was due to the incorrect mass entered into SLB. In this case OMB recognized that the response at the input mass was less than the administrator established minimum to permit purification, and the prep LC−MS run was "skipped". The following morning, the chemist corrected the mass in the Sample List, reprocessed the preprep LC−MS data, executed the prep LC−MS run, and successfully purified the product. All products were >95% purity.

Conclusions

We have described an improved approach to automated compound-specific method optimization for preparative LC−MS purification. This approach is easier to implement, more rugged, and more flexible than previously reported methods. The optimized prep LC methods provide significantly better chromatographic resolution and, hence, better compound purity than can be obtained with generic prep LC methods in the same amount of time.

The combination preprep LC−MS/prep LC−MS instrument configuration and protocols presented here are specifically designed to meet the needs of open access purification in a discovery chemistry environment. The approach should be effective in high-throughput situations as well. However, performing the preprep LC−MS analysis and prep LC−MS purification on the same platform does limit throughput. Using the methods described here, the maximum throughput is ∼96 compounds/day. For high-throughput operation, a configuration utilizing parallel preprep LC−MS and prep LC−MS systems might be more effective.[10]

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