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One-Minute Full-Gradient HPLC/UV/ELSD/MS Analysis to Support High-Throughput Parallel Synthesis

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High-throughput parallel synthesis of library compounds for early drug discovery requires high-throughput analytical methods to confirm synthesis, identify reaction products, and determine purity. An ultrafast 1.0-min HPLC/UV/ELSD/MS method was developed and compared to our standard 2.5- and 5.0-min methods in order to determine if the faster method was appropriate to evaluate compound synthesis and determine purity. In addition to using standard test mixtures, a 400-member library produced by high-throughput parallel synthesis was used for comparing the various methods. Mass spectrometric detection was used for compound identification, while UV and ELSD data offered purity assessment. Compared to our longer separations, chromatographic separation achieved using the 1.0-min method was sufficient for compound evaluation and purity assessment. This ultrafast 1.0-min HPLC/UV/ELSD/MS method is expected to increase analytical throughput tremendously, provide important information faster, and reduce the overall cycle time from synthesis to screening.

Introduction

Split-and-combine combinatorial chemistry capable of generating mixtures of hundreds of thousands analogues has been a major catalyst in changing modern drug discovery.[1−3] Given a molecular structure or scaffold that can be modified in two or more distinct positions using a variety of different reagents to add diversity elements, the fundamental tenet of combinatorial chemistry is to make all possible combinations of all diversity elements. If the basic molecular structure is an appropriate ligand for any individual biological target, then screening large numbers of small molecule analogues against the target increases the probability of discovering hits or leads proportionally. Although the premise of using a small number of reagents to make a large number of closely related analogues in order to increase the probability of identifying hits and leads is a relatively simple concept and a straightforward chemistry issue, the major challenge has always been identifying and assessing the individual compound synthesis. Split-and-combine synthesis, which generates a small number of large analogue pools, was an approach to find reasonable balance between the prebiological screen and postscreen effort. In recent years, the pharmaceutical industry has once again shifted its focus from biological screening of mixtures to screening single well-characterized compounds. The shift to move away from mixture screening is primarily due to the time-consuming and cumbersome decoding process after hit generation. Moreover, the interference and potential synergistic response from the mixtures may complicate the screening processes and make data interpretation more complex and difficult. Last, the lack of information regarding which analogues are active and which ones are not obscures the structure activity relationship (SAR) information. Because of advances in high-throughput parallel synthesis automation, combinatorial chemistry has been adapted to rapid generation of a large number of individual compounds; however, to ensure synthesis validity, assess purity/quantity and develop valid and informative SAR, all library compounds must be characterized. Because the synthesis is inherently "parallel", whereas most appropriate analyses are "serial," analytical characterization of libraries can quickly become the bottleneck in the whole process. To keep up with the speed of library synthesis, a high-throughput analytical characterization method is required.

In developing an appropriate high-throughput analytical method, two important characteristics of library compounds that must be assessed are target compound identification and purity estimation. Mass spectrometric (MS) detection is very specific in providing molecular weight information of compounds, and most of the time this information is sufficient for the synthetic chemists to verify whether they have succeeded in making the intended products. Because of its short analysis time (typically 20−60 s/sample) and the simplicity of use and interpretation of data, mass spectrometric detection using flow injection analysis (FIA) has been frequently employed to confirm combinatorial library synthesis and identify fractions of interest after purification.[4−6] More recently, in an effort to maximize the use of costly mass spectrometers, incorporation of a multiple-probe autosampler in which eight samples were analyzed in 1 min[5] has been documented for FIA/MS.

Purity estimation based on spectral peak intensity using FIA/MS has been used and reported in the literature.[6] However, ionization using an atmospheric pressure ionization

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(API) source strongly depends on the functional groups in the compounds. In addition, some solvent compositions (e.g., DMSO) and coelution of compounds may significantly suppress the ionization of the expected products[4,7] and interfere with target identification. Although FIA/MS may provide confirmation of synthesis, it typically is unreliable for purity assessment. Thus, a generic chromatographic separation in conjunction with mass spectrometric detection would be ideal. "Universal" high-throughput gradient elution chromatography with analysis times ranging from 4 to 10 min have been reported by several groups.[8−13] To match the speed of "parallel" synthesis with traditional "sequential" analysis, Kiplinger et al.[13] reported the use of dual columns to increase throughput. In this work, two columns were used alternatively for HPLC/MS analysis using a 10-port switching valve so that one of the columns was used for analysis while the other column was regenerated. They reported a cycle time of 8 min/sample using this dual column HPLC/MS method. Zeng et al.[14] used the parallel operation of two HPLC columns coupled with a dual sprayer interfaced to a mass spectrometer in support of combinatorial libraries. This approach effectively doubled their analytical capacity. Mass spectral signals for the two samples were acquired simultaneously in one MS data file, while two individual UV traces were generated. The total run time was 4.2 min for every two samples. The effort in our group to reduce the analysis time even further resulted in evaluation of several fast gradient HPLC/UV/MS analyses employing a 4.6 × 30 mm column.[15−17] It was shown that a total analysis time of 1.0 min/sample could be achieved by HPLC/UV with sufficient resolving power for the characterization of samples generated by high-throughput parallel synthesis.

Chromatographic purity assessment requires a detection technique that has universal responses for all classes of compounds. Refractive index and low wavelength UV detection techniques are considered to be close to the ideal universal detector. However, refractive index detection is not suitable with solvent gradients. Low-wavelength UV is preferred to refractive index because of its sensitivity and gradient elution compatibility. Nevertheless, UV absorption depends on the presence of chromophores in the compound, and it may not respond to entities without any strong chromophores. Use of another detection technique in addition to low-wavelength UV would be highly desirable. Evaporative light scattering detection (ELSD) is based on light scattering from solute particles and is known to provide reasonably similar response for closely related compounds or of similar classes.[18] Chemiluminescent nitrogen detector (CLND) in conjunction with HPLC/UV/MS[19] has also been used to identify, quantify, and determine the purity of library compounds. Although CLND can produce a linear generic calibration curve, this technique requires nitrogen-containing compounds and non-nitrogen solvents. The technique of HPLC/UV/CLND/MS needs to be further evaluated in terms of cost, high throughput, ruggedness, and qualitative as well as quantitative analysis.

In this paper, we will discuss ArQule's continued effort to push the limit of high-speed separation and increase analytical throughput for the characterization of parallel synthesis combinatorial libraries. A major consideration for this work was to assess approximate purity of expected products using various detection techniques without increasing the probability of false positive or negative results due to compromised separation conditions.

Experimental Section

I. Chemicals. HPLC grade water, acetonitrile, and dimethyl sulfoxide (DMSO) were purchased from J. T. Baker (Phillipsburg, NJ). Formic acid was obtained from EM Science (Gibbstown, NJ). The following chemicals for the test mixture were purchased from the vendors specified and used without further purification: chlortetracycline from Aldrich (St. Louis, MO), *N*-[4-(4-aminobenzyl)phenyl]-5-norborene-2,3-dicarboximide (APND) from Aldrich, dibucaine from Sigma (St. Louis, MO), rhodamine B from Fluka (Milwaukee, WI), flavone from Fluka, and 2-(2*H*-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol (BTMBP) from Aldrich. The concentrations of the compounds dissolved in DMSO were as follows: 0.4 mg/mL of chlortetracycline, 0.1 mg/mL of APND, 0.2 mg/mL of dibucaine, 0.1 mg/mL of rhodamine B, 0.1 mg/mL of flavone, and 0.2 mg/mL of BTMBP. The HPLC test standard used to show the impact of equilibration time consisted of 2-acetamidophenol, 2-hydroxydibenzofuran, and 3-(4-*tert*-butylphenoxy)benzaldehyde from Aldrich, each at a concentration of 1 mg/mL in DMSO.

II. Instrumentation. The schematic diagram of the instrumentation is shown in Figure 1. The experimental setup consists of a liquid handler for injection of samples from a 96-well plate format, an HPLC with UV detection, an evaporative light scattering detector (ELSD), and a mass spectrometer (MS). Upon sample injection, the liquid handler sends out a start signal to the HPLC and the MS, triggering gradient elution and mass spectral acquisition (trigger signal transfer not shown in Figure 1). The UV signal is autozeroed at this point, and simultaneously, the HPLC controller sends out a signal to trigger ChromPerfect data acquisition. UV and ELSD signals are acquired simultaneously by two different acquisition software programs, ChromPerfect (Justice Innovations, Mountain View, CA) and MassLynx/OpenLynx (Micromass, Manchester, U.K.) on the mass spectrometer.

A. Sample Injection. A Gilson 215 liquid handler (Gilson, Inc., Middleton, WI) equipped with a model 819 injection valve actuator was used for sample injection to handle samples submitted in vials or 96-well plates. The liquid handler can accommodate up to 12 96-well plates. The injection needle and injection port were rinsed automatically with acetonitrile between injections. Samples were dissolved in dimethyl sulfoxide (DMSO), and the injection volume was between 2 and 5 μL. For the reequilibration study, a manual Rheodyne injector was used, as none of the integrated commercially available autoinjectors was able to inject fast enough after the end of a run.

B. High-Performance Liquid Chromatography. A Shimadzu HPLC system (Columbia, MD) consisting of two LC-10ADvp pumps, a DGU-14A degasser, an SCL-10Avp system controller, and an SPD-10Avp UV detector was used throughout the experiment. Two pumping units were em-

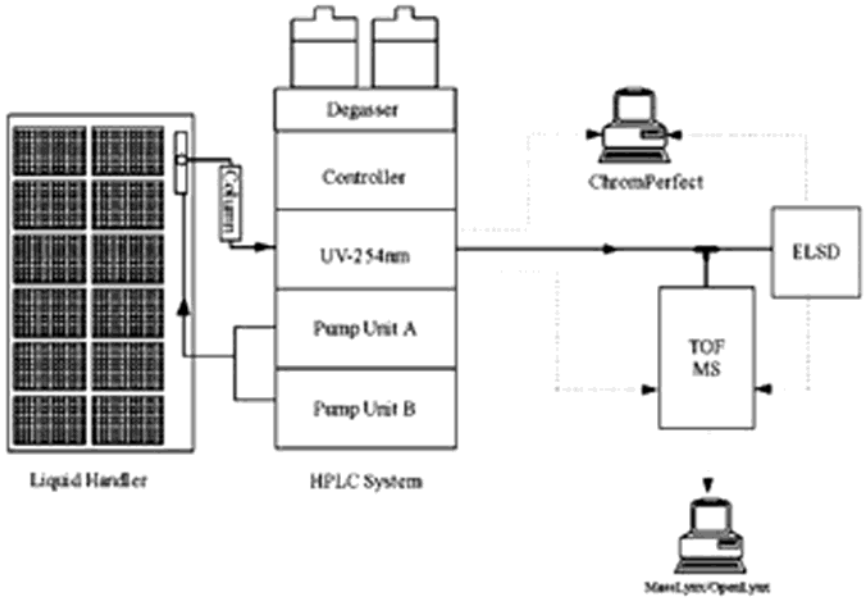

Figure 1. Schematic diagram of experimental setup. Solid line represents liquid flow, and dotted line represents signal transfer.

ployed for a high-pressure gradient system to minimize time lag and gradient delay, thus achieving ultrafast gradient HPLC/MS. Connections between the injector, the column, and detectors were made using 0.005-in.-i.d. PEEK tubing, except for the interface connection to the mass spectrometer. In order to minimize band broadening and to restrict the flow entering the mass spectrometer, 0.0025-in.-i.d. PEEK tubing was used between the split tee and the mass spectrometer. A Zorbax SB-C8 column (4.6 × 30 mm, 3.5-μm particles) was purchased from MacMod (Chaddsford, PA). Water and acetonitrile with 0.1% (v/v) formic acid were used as solvents A and B, respectively. Solvent gradients from 15% B to 95% B were applied in 0.7, 1.75, and 3.5 min at a flow rate of 4, 3, and 2 mL/min, for 1.0-, 2.5-, and 5.0-min methods, respectively. UV absorbance was monitored at 254 nm.

C. Evaporative Light Scattering Detector. A Sedex 55 evaporative light scattering detector (SEDERE, Alfortville, France) was coupled with the HPLC system after UV detection through a split tee (Figure 1). Approximately 90% of the total flow entered into ELSD. Air pressure used for the ELSD nebulizer was at 1 bar, and the temperature was set at 40 °C. The factory unit was modified in-house by removing the electronic time filter, thus allowing rapidly eluting peaks to be monitored effectively

D. Mass Spectrometry. An orthogonal reflectron time-of-flight mass spectrometer (LCT, Micromass, Manchester, U.K.) was coupled with the HPLC system through a split tee (Figure 1). Approximately 10% of the total HPLC flow was delivered to the mass spectrometer operated in the positive electrospray mode throughout the study. Mass spectral data were acquired from 190 to 800 amu at a data acquisition rate of 0.2, 0.3, and 0.5 s/spectrum for 1.0, 2.5, and 5.0 min methods, respectively. The resolution of the mass spectrometer was set at 5000.

III. Data Processing for Library Samples. Library samples were obtained in 96-well microtiter plates. Pertinent sample information was transferred to a custom application program developed in-house to control the Gilson liquid handler. This software initiated the Gilson injector to rinse and aspirate the next sample before the end of a current run in order to reduce the overall cycle time. Mass spectral data were acquired and then automatically processed by OpenLynx Diversity (Micromass). UV and ELSD data were acquired simultaneously by ChromPerfect and MassLynx. Peak integration of the UV and ELSD chromatograms was performed using OpenLynx and ChromPerfect independently, and the results were compared afterward.

Results and Discussion

Several years ago, all lead optimization library compounds produced at ArQule were analyzed using a 30-s "pseudo" HPLC/MS[7] method to confirm the synthesis and a 5.0-min HPLC/UV/ELSD method to assess purity. Similarly, ~25% of each lead generation library was analyzed using a similar approach. Moreover, the independent "pseudo" HPLC/MS and 5.0 min HPLC/UV/ELSD analyses were validated by a select number of independent HPLC/UV/ELSD/MS analyses. This approach was developed in order to maximize the number of QC analyses while minimizing the number of HPLC/MS systems and therefore maintaining a cost-effective operation. Our continuous efforts to develop faster chromatographic separations have allowed us to reduce the overall analysis time, and for the past few years, we have been using a 2.5-min HPLC/UV/ELSD/MS analysis method for characterization of all samples synthesized without the need to add more instrumentation. Recognizing that the need for rapid analysis will continue as high-throughput parallel synthesis becomes a more important component of early drug discovery, we have investigated the possibility of reducing the analysis time further while still obtaining sufficient separation for compound identification and purity assessment. A 1.0-min HPLC separation in combination with UV, ELSD, and MS detection was developed and compared to our 2.5- and 5.0-min analyses.

Minimizing gradient delay volume and extra-column dead volume was critical to the success of the ultrafast gradient

method. A high-pressure mixing solvent delivery system with two pumping units was chosen to provide maximum control of the gradient profile. The system volume between the mixing point of the two solvents and the column is defined as the gradient delay volume. The gradient delay volume must be minimized, so the actual gradient observed at the column is very close to the programmed gradient profile. To achieve this goal, small-i.d. tubing was used to connect the mixer, sample loop, and column. The tubing length between connections was minimized, and a low-volume injection loop (10 μL) was employed. A small internal volume solvent mixer (2 μL) was used for this setup. This configuration allows effective control of elution conditions for separation and column reequilibration.[8]

Using short columns packed with high-performance small particles, very efficient separations are possible. This means that the peak volumes are small (on the order of tens of microliters), which in turn makes the system very sensitive to extra-column dead volume. A general rule to achieve good system performance is keeping the dead volume below 20% of the peak volume. For this type of application, sensitivity is usually not an issue, and larger-i.d. columns generate larger peak volumes and, therefore, reduce the risk of a loss in resolution due to extra-column band broadening. We routinely use 4.6-mm-i.d. columns for this type of analysis, whereas LC/MS applications that require higher sensitivity are usually employing smaller-i.d. columns. We use 0.005-in.-i.d. tubing postcolumn for low-volume connections and employ a semimicro UV flow cell with low volume (2.5 μL). Behind the UV detector, we continue with 0.005-in. tubing to connect and split the flow to the MS and the ELSD. Observing these guidelines ensures optimized peak shapes, which results in fast separations with very good performance.

The peak width observed in the fast gradient HPLC/MS methods ranged from ~1 to 3 s on UV traces for the 1.0- and 5.0-min analyses, respectively. To capture the very narrow chromatographic peaks, a mass spectrometer with a fast acquisition capability was required. A time-of-flight mass spectrometer was identified for this application because it is not a scanning-dependent device, such as quadrupole-type instruments. Instead, ion signals with respect to the flight time are recorded in a time-of-flight mass spectrometer, which is usually in the order of hundreds of microseconds. Therefore, the limiting factor for acquisition speed in the time-of-flight mass spectrometer system is not its "scanning" speed, but its ability to detect, convert, and save the signal onto the hard disk, which depends on the speed of the time-to-digital converter (TDC) and the acquisition computer. The maximum acquisition speed of the LCT instrument is 0.1 s, as specified by manufacturer, but the chromatograms acquired at this speed were found to have low signal-to-noise (S/N) ion currents and unexpected spikes. Thus, an acquisition rate of 0.2 s was used for the fastest separations. The acquisition software of the time-of-flight mass spectrometer (MassLynx version 3.2, Build 004) acquired analogue signals at the same sampling rate as the mass spectral signal; therefore, the sampling rate for the analogue channel depended on the mass spectral acquisition speed. As the gradient slope became steeper, the chromatographic peak width was compressed down to ~1 s for the 1.0 min analysis. Since we intended to use the analogue channel data (UV and ELSD) for purity assessment, an accurate representation of the real peak shape was essential for the peak integration process. With a 0.2-s acquisition time and a peak width of 1 s, the number of data points across a peak is 5−7, which is considered relatively low for proper peak shape representation. Thus, a stand-alone software package, ChromPerfect, was utilized for UV and ELSD data acquisition in parallel with MassLynx. ChromPerfect acquired the UV and ELSD data at the rate of 30 points/s, which should be sufficient for the narrowest peak observed in this study. OpenLynx Diversity was used to process total ion current (TIC), UV, and ELSD data, automatically generating a report indicating "Yes" and "No" for the presence or absence of the target compounds, and provided percentages of the peak area for the corresponding UV and ELSD peaks. ChromPerfect simultaneously integrated the UV and ELSD signals and generated percentage peak area reports without target identification information. The reports of peak area integration from ChromPerfect were used to evaluate the reliability of MassLynx analogue channel data for peak area integration.

Three gradient analyses designated as 1.0-, 2.5-, and 5.0-min methods were tested using a standard test mixture and a 400-member library prepared by high-throughput parallel synthesis. The results were compared in terms of target compound identification and purity estimation.

I. Evaluation of the Methods Using a Standard Test Mixture. A standard test mixture with compounds of various retention times was prepared to evaluate the separation achieved by each method. The 1.0-, 2.5-, and 5.0-min HPLC/UV chromatograms processed by MassLynx/OpenLynx are shown in Figure 2 using the same time scale in order to contrast the run times and the peak width difference between the methods. The peaks labeled as 1, 2, and 3 are isomers of chlortetracycline. APND, dibucaine, rhodamine B, flavone, and BTMBP elute in the order indicated by the labels 4−8, respectively, in Figure 2. All three methods are capable of baseline separation of the test mixture. The small peak observed at 1.45 min in the 5.0-min analysis was an impurity originating from rhodamine B. This impurity was still resolved by both the 2.5- and the 1.0-min methods, indicating that there is enough separation capacity using the small columns, if the system is appropriately configured, to meet the analytical expectations required by parallel synthesis techniques. The individual mass spectra of the resolved peaks from the 1.0-min separation are shown in Figure 3, and the overall quality of these spectra are similar to those obtained from the 5.0 min analysis. Since each peak was baseline-resolved, the molecular ion for each compound was clearly observed without cross contamination from other peaks.

The UV and ELSD chromatograms acquired by MassLynx for the three methods and normalized to each time scale are shown in Figure 4. These chromatograms are qualitatively similar to the corresponding chromatograms acquired using the ChromPerfect software. Gradient delay can be estimated from the UV signal baseline. For example, in Figure 4A, the solvent front (corresponding to unretained compound elution) appeared at 0.1 min, and the baseline slowly

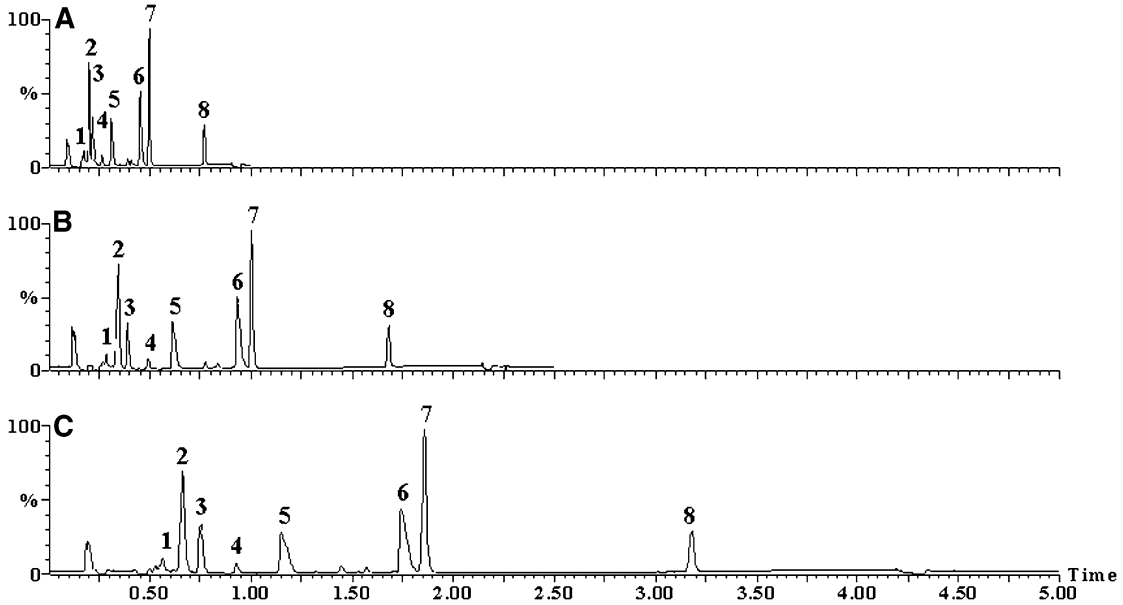

Figure 2. HPLC/UV chromatograms of (a) 1.0-, (b) 2.5-, and (c) 5.0-min methods for the standard test mixture. Peaks labeled as 1, 2, and 3 are chlorotetracycline isomers; 4 is APND; 5 is dibucaine; 6 is rhodamine B; 7 is flavone; and 8 is BTMBP.

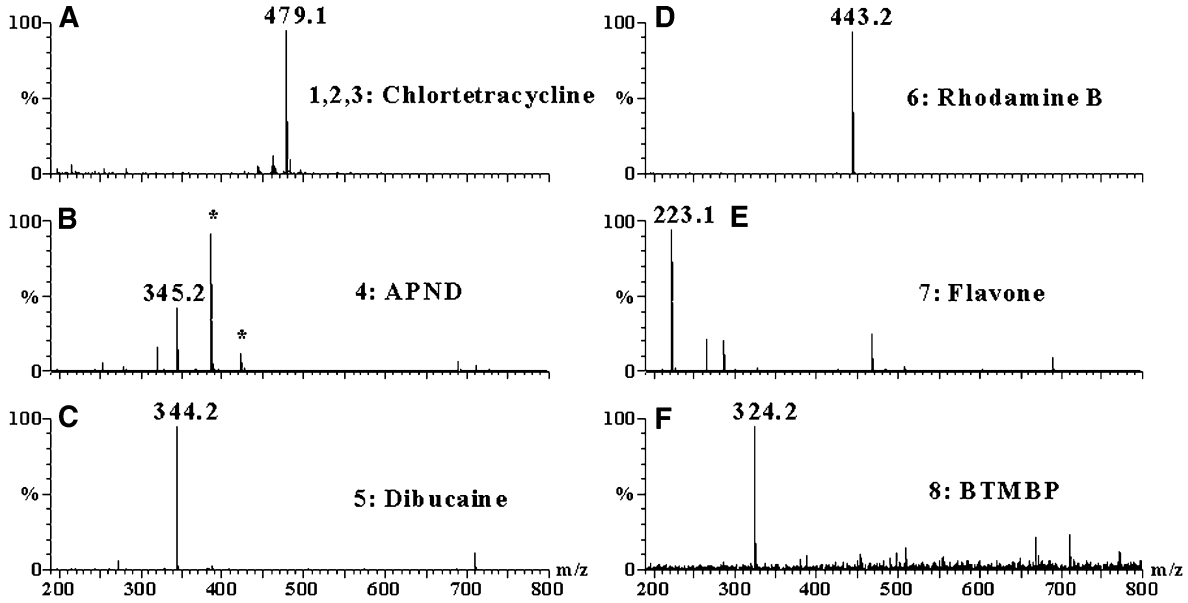

Figure 3. Mass spectra of standard test mixture compounds obtained by 1.0-min method: (a) chlortetracycline, (b) APND (peaks marked with an asterisk are acetonitrile and DMSO adducts of the molecular ion), (c) dibucaine, (d) rhodamine B, (e) flavone, and (f) BTMBP.

increased until 0.91 min as a result of percentage increase in acetonitrile and dropped down at that time, indicating the end of the gradient. The 1.0-min method was programmed to start at 15% B, increase to 95% B in 0.7 min, hold at that concentration for 0.1 min, and return to 15% B in 0.1 min, which indicates that the gradient ended in 0.9 min. Comparing the programmed gradient to the observed one in Figure 4A indicates that the gradient delay for this method was 0.1 min. The gradient delays for the 2.5- and 5.0-min methods were observed to be 0.14 and 0.20 min, respectively, using the corresponding UV traces. Since the only difference between methods which could affect the gradient delay is the flow rate, these values are rational considering that the flow rates of 4, 3, and 2 mL/min were used for 1.0-, 2.5-, and 5.0-min analyses, respectively. The total system volume (up to the UV detector, including column void volume) was estimated to be $\sim$400 μL. Considering that the empty column volume is 0.5 mL and the porosity of silica gel is $\sim$75% further corroborates the estimated system volume value.

The average baseline peak width (measured as full width at 10% height) of the HPLC/UV chromatograms was 1.0, 1.8, and 3.1 s for 1.0-, 2.5- and 5.0-min methods, respectively. To compare separation efficiency between the methods, the peak capacity was compared. The peak capacity, defined by the gradient time divided by average baseline peak width, was approximately 42, 58, and 68 for the 1.0-, 2.5-, and 5.0-min analyses, respectively. The column volume is 0.5 mL for a 4.6 $\times$ 30 mm column, and the gradient volume can be calculated from the flow rate and gradient time for each method. Empty column volume is typically used to calculate gradient volume, since it is hard to estimate the actual column volume of packed columns. The separations

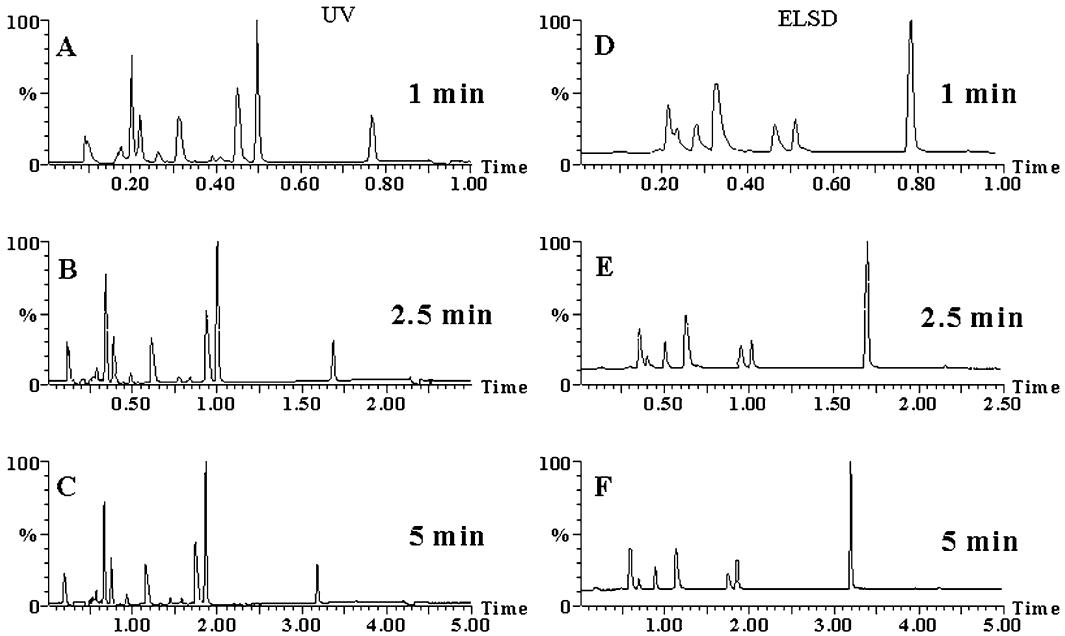

Figure 4. Normalized HPLC/UV chromatograms of standard test mixture with (a) 1.0-, (b) 2.5-, and (c) 5.0-min methods. Normalized HPLC/ELSD chromatograms of standard test mixture with (d) 1.0-, (e) 2.5-, and (f) 5.0-min methods.

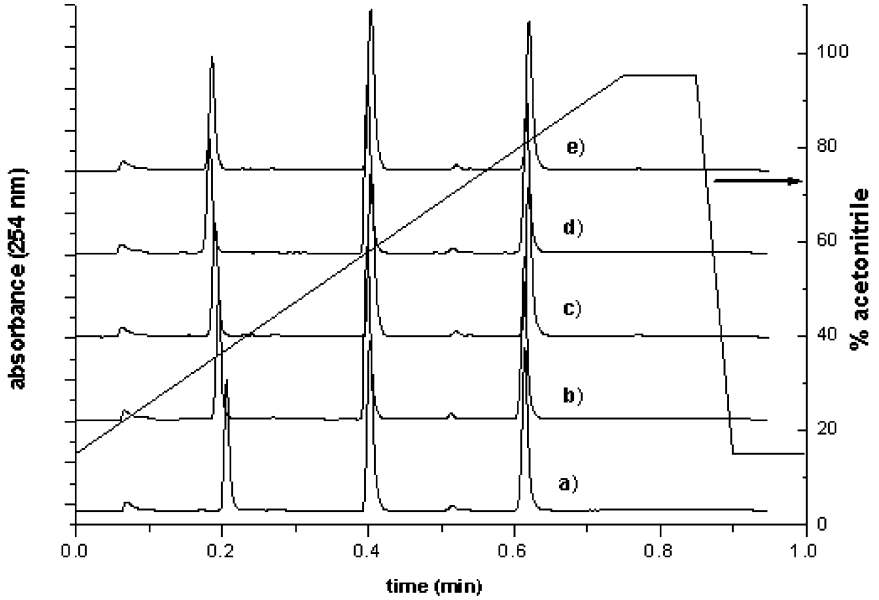

Figure 5. Impact of equilibration time on retention times for the 1-min separation. Gradient profile is shown overlaying the chromatograms. HPLC test standard is injected after (a) 0.1 min at initial conditions (0.67 column volumes), (b) 0.2 min at initial conditions (1.35 column volumes), (c) 0.3 min at initial conditions (2 column volumes), (d) 0.4 min at initial conditions (2.67 column volumes), and (e) 0.6 min at initial conditions (4 column volumes).

shown in Figure 4 were achieved by ~5.6, 10.5, and 14 column volumes during the linear gradient of 1.0-, 2.5-, and 5.0-min methods, respectively. A typical starting point considered for gradient separation is 10 column volumes,[20] which is close for all three methods.

The impact of the equilibration time necessary to achieve consistent elution times is shown in Figure 5. The time that the system is flushed with starting eluent composition is progressively increased between runs, and the elution times are monitored with the three compounds of the HPLC standard covering the elution range of the gradient. As can be seen from the overlay of the chromatograms, ~0.4 min of reequilibration (~2.7 column volumes) is completely sufficient to ensure consistent retention times for all three components of this mixture (Figure 5d). While the experiment had been extended out to 4 min of equilibration time (data not shown), the elution times remained consistent after 0.6 min of equilibration. Since the gradient employed returns to initial conditions at 0.9 min (see the inserted gradient profile), an overall cycle time of 1.3 min, or less than 1 min 20 s, can be achieved. By initiating the next sample injection before the end of a current run, as described in the Experimental Section, an injection-to-injection cycle time of ~1 min 20 s can be practically realized in a high-throughput approach, allowing the required 0.4 min of reequilibration time without additional extension of cycle time. Analysis of the standard test mixture using the 1.0-min gradient method with a 1-min 20-s cycle time showed good reproducibility

Table 1. Comparison of UV and ELSD Peak Integration (Area %) for Standard Mixture Using OpenLynx and ChromPerfect for the 1.0-, 2.5-, and 5.0-Min Methods

	UV						ELSD					
	OpenLynx			ChromPerfect			OpenLynx			ChromPerfect		
compound	1.0-min	2.5-min	5.0-min	1.0-min	2.5-min	5.0-min	1.0-min	2.5-min	5.0-min	1.0-min	2.5-min	5.0-min
chlortetracycline	31.5	31.9	29.1	32.5	32.6	31.6	23.2	20.0	20.7	23.6	23.4	22.0
APND	1.4	1.4	1.3	1.5	1.4	1.2	4.1	5.2	4.3	5.4	5.1	4.3
dibucaine	14.0	14.0	14.6	13.6	14.0	13.8	29.0	24.3	21.6	30.0	23.6	21.4
rhodamine B	15.3	15.7	16.8	15.5	16.0	15.8	6.3	6.9	5.8	6.5	6.7	5.9
flavone	26.5	26.5	27.4	24.2	25.4	25.5	7.1	7.9	7.3	6.7	7.6	7.0
BTMBP	9.3	9.0	9.5	8.6	8.6	8.8	30.2	35.6	40.3	27.9	33.6	39.5

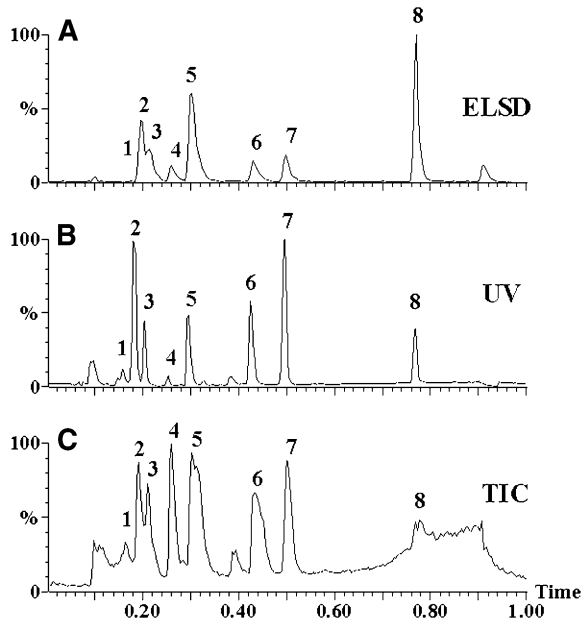

Figure 6. 1.0-min HPLC/MS analysis of standard test mixture: (a) ELSD, (b) UV, and (c) TIC.

of the retention time with <2% RSD for 20 repetitive injections. These data contradict the recommended 10-column volumes for column regeneration[20] expectation of traditional gradient chromatography; however, the very short column length employed in this study was not available when these empirical rules were generated.

Representative TIC, UV, and ELSD data acquired by the 1.0-min method for the test mixture are shown in Figure 6. Although the band broadening of the chromatographic peaks on TIC and ELSD, as compared with UV, is apparent, the separation achieved still allows correlation of chromatographic peaks between different detection modes. The time shift between detection modes is due to the physical distance of the ELSD and the MS from the UV and the delay experienced by the ELSD from spraying of the sample through evaporation of the solvent to final detection (in the order of 4−6 s). The delays are consistent within the gradient, and the software is programmed to align retention times for the different traces. The band broadening of the TIC chromatogram, in addition to extracolumn effects, enhanced by the reduced split-flow, may be due in part to the sensitivity and dynamic range differences between the detection methods. In this case, the ion signal exceeded the mass spectrometric detection dynamic range, resulting in the signal saturation and peak broadening. Use of smaller injection volumes, which were more appropriate for mass spectral signal acquisition, produced very weak ELSD response. Since UV and ELSD signals were to be integrated for purity estimation, injection volumes, which provided strong signals for UV and ELSD, were used. OpenLynx and ChromPerfect were used to integrate the UV and ELSD data of the test mixture for all three methods independently to assess purity, and the results are summarized in Table 1. The results indicate that the percentages of peak areas were comparable among the three analysis methods and between the ChromPerfect and OpenLynx software packages.

II. Evaluation of the Methods Using Combinatorial Library Samples. In general, a library sample generated by parallel synthesis is a relatively simple mixture of residual starting material and possible side products as well as the intended product. The standard test mixture consisted of six diverse compounds and generated eight chromatographic peaks. Eight peaks in one chromatogram would be considered a worst-case scenario in parallel library synthesis, since a large number of peaks would indicate that the reaction was not optimized or the yield of the intended product could be low. The three fast gradient methods were further evaluated using a small ArQule parallel synthesis library consisting of 400 individual samples to validate the ruggedness of the methods as well as product identification and purity estimation.

Total run time for this 400-member library was <8 h using the 1.0-min method, as compared with 17 and 34 h for the 2.5- and 5.0-min methods, respectively. A single column was used throughout the entire study with more than 1200 sample injections, including test mixture analysis, and no chromatographic degradation was observed. Moreover, long-term studies conducted in our laboratory have shown that these columns can be used for more than 10 000 individual analyses using the described conditions.

OpenLynx was used for automated data processing to generate typical pictorial 96-well microtiter format reports with green and red dots to indicate the presence or absence, respectively, of the expected molecular mass. The results of the representative 400-member library analyzed using the three different analytical methods correlated well with each other. Only three samples from one plate gave results using the 5.0-min method that were different from either the 2.5- or 1.0-min method. Closer examination of these conflicting data revealed that the molecular ions were present in the data acquired with all three methods but with marginal intensity around the user-set threshold. Thus, a sample may

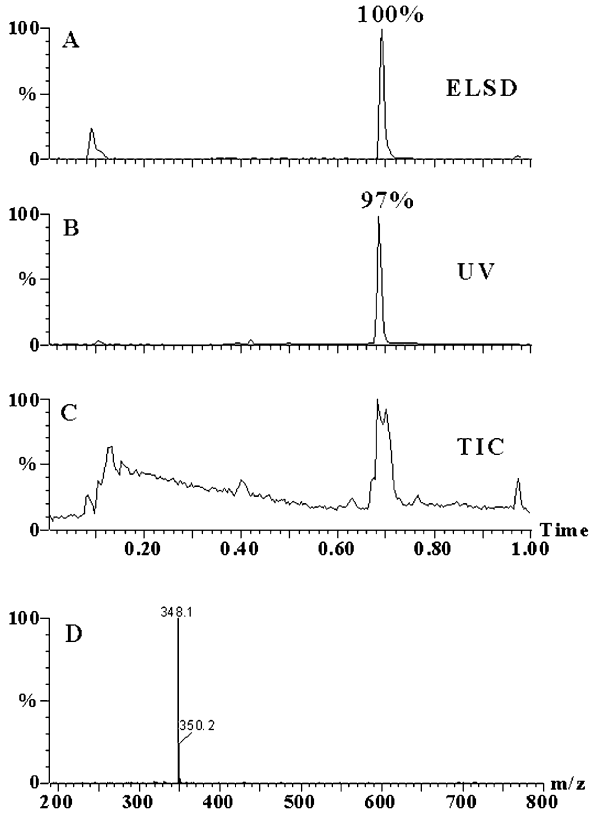

Figure 7. 1.0-min HPLC/MS analysis of a representative successful library sample synthesis: (a) ELSD, (b) UV, and (c) TIC (d) mass spectrum of the main peak.

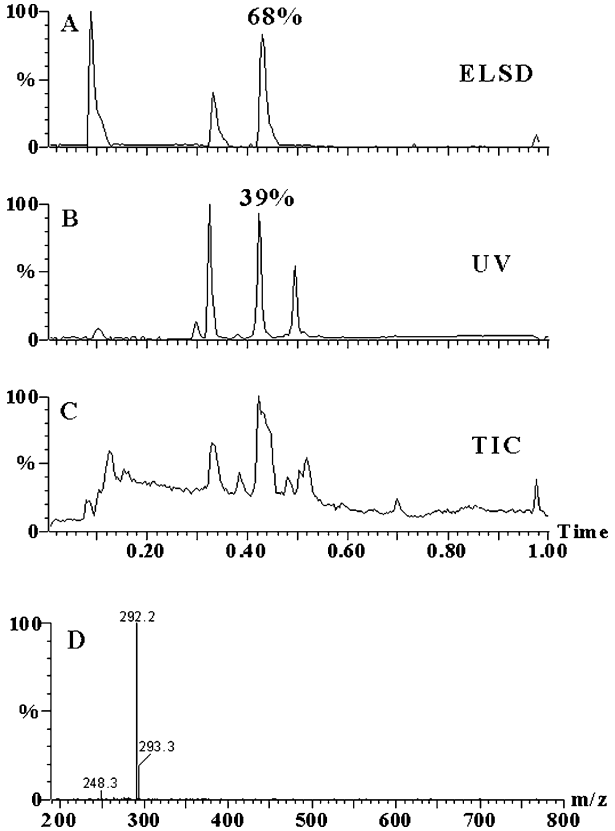

Figure 8. 1.0-min HPLC/MS analysis of a representative failed sample synthesis: (a) ELSD, (b) UV, and (c) TIC (d) mass spectrum of target compound peak.

have been identified as positive with one analysis but negative with another. The data for a typical example of a library sample is shown in Figure 7. Both UV and ELSD chromatograms show >95 % purity, and the mass spectrum of the main peak (Figure 7D) indicates the expected mass of the product. The presence of one chlorine atom in the compound, evident from the isotopic pattern, further confirms the expected structure. Another example from this library (Figure 8) contained side products in addition to the intended product, with significant intensity on both UV and ELSD detection. The peak at retention time of 0.42 min was identified as the intended product by the presence of the expected molecular weight indicated in Figure 8D. Although this sample did not meet the acceptable purity criteria, the 1.0-min analytical method easily identified this sample as a failed synthesis. The relative difference in reported purity between detectors for this example further underscores the need for purifying synthesized compounds in order to avoid having to determine which detector represents reality more appropriately.

Peak integration of UV and ELSD chromatograms for the three methods was performed independently using OpenLynx and ChromPerfect. Table 2 is a representative subset of the UV sample purity for the first 20 samples in the library. Figure 9 is a plot of the ChrormPerfect UV data obtained for the 1.0-min method relative to the 5.0-min method, with a correlation coefficient of 0.9546. Similarly, a plot of the 1.0-min UV data obtained by OpenLynx relative to Chrom-Perfect is shown in Figure 10 with an associated correlation coefficient of 0.9712. The reported purity for each sample

Table 2. Comparison of UV Peak Integration for 1.0-, 2.5-, and 5.0-Min Methods Using OpenLynx and ChromPerfect for Representative Library Samples

	1.0-min		2.5-min		5.0-min	
sample	Open-Lynx	Chrom-Perfect	Open-Lynx	Chrom-Perfect	Open-Lynx	Chrom-Perfect
A0101A2	95.4	91.2	100.0	97.0	100.0	92.0
A0101B2	73.1	72.3	76.6	69.1	80.8	71.9
A0101C2	93.5	93.1	95.8	95.5	95.6	94.7
A0101D2	91.9	92.0	84.7	84.5	94.8	93.4
A0101E3	65.9	63.9	57.9	54.4	68.9	64.0
A0101F3	89.5	87.8	89.0	79.2	87.1	79.6
A0101G3	82.5	68.7	77.5	66.8	84.5	80.4
A0101H3	100.0	95.6	96.6	96.0	100.0	95.3
A0101A4	85.0	83.2	91.7	83.5	92.8	83.2
A0101B4	39.2	38.9	38.5	36.2	39.3	37.3
A0101C4	85.6	84.7	84.1	83.3	85.9	83.2
A0101D4	75.1	72.1	80.3	76.2	80.8	76.5
A0101E5	83.6	78.4	71.5	69.4	75.4	71.1
A0101F5	86.6	82.9	87.0	84.8	86.4	82.5
A0101G5	93.6	86.9	87.0	85.3	87.6	83.7
A0101H5	89.9	87.5	89.1	87.4	88.6	87.4
A0101A6	92.6	84.4	87.2	84.4	86.1	82.6
A0101B6	77.6	72.3	75.9	72.9	79.1	75.3
A0101C6	96.8	95.1	90.2	89.9	96.6	93.9
A0101D6	93.7	90.9	94.2	91.0	100.0	93.2

was comparable among the three methods and between OpenLynx and ChromPerfect, suggesting that the 1.0-min method provided sufficient separation for the target analysis. Close examination of the data indicates that minor peaks and shoulder peaks are not as well-defined by OpenLynx and usually not integrated properly, thus accounting for the minor differences in the reported purity between data acquisition

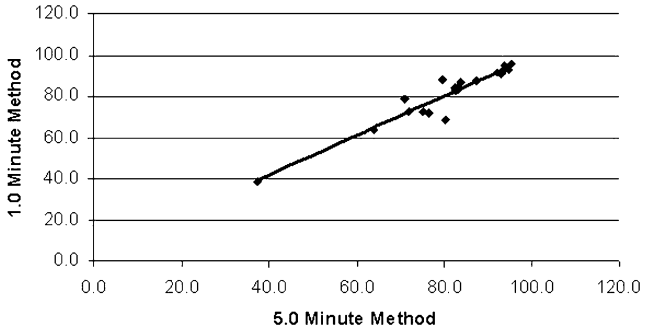

Figure 9. Correlation of UV purity data for representative library subset obtained with the 1.0- and 5.0-min methods.

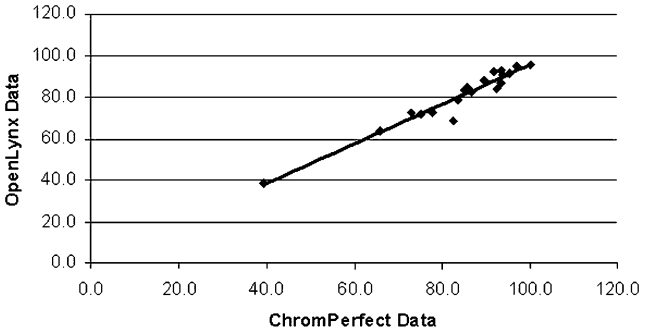

Figure 10. Correlation of 1.0-min UV purity data acquired with OpenLynx and Chromperfect for a representative library subset.

software. This limitation is due to the fact that the number of data points acquired was not enough to adequately define the peaks in a small percentage of the samples. In general, however, using a proper delay time to align the chromatograms from different detection modes, OpenLynx can correlate UV or ELSD peaks with the intended compound and adequately estimate purity.

Conclusion

To keep up with high-throughput parallel synthesis of library compounds to support early drug discovery, our routine 2.5-min separation time using a generic fast gradient method was further reduced by using a steeper gradient elution at a higher flow rate. The 1.0-min method demonstrated sufficient separation efficiency for library characterization. This ultrafast method increases the sample throughput more than 2-fold, maximizes the use of costly mass spectrometers, and reduces solvent consumption per sample. However, to capture very narrow chromatographic peaks, a mass spectrometer with high acquisition speed was required. Multidimensional detection employing UV, ELSD, and MS provides a more powerful analytical tool for library characterization rather than relying on a single detection mode, which could be misleading. Although the evaluation presented in this report suggests that five to seven data points across a peak were acceptable for peak integration, more complex reaction mixtures where components are unresolved and appear as shoulders on a main peak would require a faster acquisition of analogue data provided by software programs, such as ChromPerfect.

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