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A novel method for immediate post-purification purity determination of fractions collected during high-throughput purification

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Abstract

Following purification, the fractions of purified samples typically are analyzed to determine the relative purities of each fraction. We report a novel technique for performing post-purification analysis immediately after each preparative LC/MS run. The Single Pass Compound Purification and Analysis System (SPACPASS) samples and stores a representative aliquot from the fraction *while it is being collected*. Demonstrated for '1:1' fraction collections, this method of fraction purity assessment streamlined sample processing by reducing post-purification sample handling. For 97% of the collected fractions, this technique provided relative purities to within ±5% when compared with more traditional post-purification analysis.

Keywords: High throughput purification and analysis; High throughput purification; Fraction analysis; Single pass purification and analysis; HPLC/MS purification; LC/MS purification; Mass-directed fraction collection; Prep LC/MS; SPACPASS; Representative sampling; Representative flow stream sampling; Purity

1. Introduction

Starting with a lead series, the goal of a medicinal chemist is to synthesize bioavailable compounds possessing the desired biological activity and the required cross-assay selectivity/specificity. With the push to continually shorten the time of each cycle in lead optimization, medicinal chemists rely heavily on automated purification systems, such as HPLC [1–5] or supercritical fluid chromatography systems (SFC) [6–9], to obtain the highly pure material needed for screening and for subsequent formation of structure activity relationships [10]. Due to its relative simplicity and history of reliability, UV-triggered purification is widely used with HPLC. With the ability to detect many drug-like compounds, the likelihood of collecting the compound of interest is generally high when using UV-triggered collection. With few exceptions [3,4,11], collecting fractions based on UV response generally results in a large number of samples [10,12,13], which may be culled using mass spectrometric data acquired during purification or through flow-injection MS analysis [5]. With resources expended collecting and tracking each fraction, it usually is more efficient to collect only the fractions with the desired compounds.

The ability to routinely inject one sample and collect one fraction was improved with mass-directed fraction collection [14], a purification technique that capitalized on the ability of a mass spectrometer to distinguish compounds based on their m/z. Since many drug-like compounds can be ionized by LC/MS, this technique for high throughput purification is gaining wider usage among medicinal chemists. Although all automated purification systems may encounter technical

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difficulties and fail to provide pure enough or even any of the desired product, when using *m*/*z* triggered fraction collection, a clogged splitter can result in the loss of a number of compounds [4] during purification and, unlike with UV-triggered purification, there may be little or no feedback as to the success with regard to collection efficiency. To address this, UV detectors have been used to monitor the waste stream after the collection valve in order to semi-quantitatively estimate the collection efficiency of well-resolved components in a solution [15]. While this estimate may be sufficient in many cases, it assumes all of the material sampled is injected and traverses the column. Due to the high concentration of the injected sample, the detector may be saturated, and, under these conditions, it would not be possible to estimate collection recovery accurately. In order to evaluate instrument performance, a more quantitative assessment relating the amount of sample collected to the amount injected is preferred because it mimics the sample handling used for HT-purification. This method involves injecting a known amount of a standard compound, collecting and evaporating the fraction, redissolving it to a theoretical concentration based on 100% collection efficiency, and comparing its response to that of a reference sample created at 100% of the theoretical concentration. Using Fmoc-Thr(tBu)-OH as a standard, recoveries from mass-directed purification systems have been reported to span the range from 83% to 98% of what is injected [16]. Since it is impractical to perform this in-depth testing prior to each purification queue, an automated, facile method is needed for assessing and, if necessary, optimizing fraction collection efficiency by quantifying a collected standard as a function of delay time between observation of the expected ion and the triggering of fraction collection.

Following purification, the fractions collected on an preparative LC/MS purification system are analyzed to determine if the material meets the purity standards required for later-stage screening in lead optimization. Such post-purification analysis can be achieved on the same instrument using an automatically-generated sample list. However, since it was reported that the fractions collected may not achieve homogeneity within 24 h [17], and since representative fraction sampling is critical to obtaining an accurate result, additional sample processing steps, e.g., sample agitation or sample evaporation and redissolution, are necessary. These added steps of manual intervention and offline sample processing delay the acquisition of the post-purification sample data, and, ultimately, the delivery of compounds for additional testing. The need for manual intervention is particularly inefficient if the purification queue is completed overnight and the instrument remains idle until the start of the next workday. Clearly a better solution would be to sample a representative aliquot from each fraction and to automatically analyze the samples. Such technology would streamline the purification/post-purification process by removing these additional steps and decreasing instrument idle time.

Similar concepts of continuous sampling and analysis have been explored in high-throughput purification. For example, it was reported that the amount of sample collected during mass-triggered purification could be estimated using the response from an evaporative light-scattering detector (ELSD) [18]. This technique relied on estimating detector response by integrating the portion of the peak corresponding to the collection event. The accuracy of the results depended not only on the 'universality' of the detector's response [19], but also on the timing between the fraction collection event and of the split flow stream reaching the ELSD.

Fraction collection and reanalysis of column eluent has been demonstrated with 2D chromatography. Fractions are collected using a set time window [20] or a set time interval [21,22]. Since all of the eluting material may be collected off-line [21] or on-line [22] and then analyzed using an orthogonal technique, 2D-LC has been used in proteomics to help increase the number of compounds detected over the 1D techniques and maintain sensitivity.

By combining these two concepts, a single-pass purification and analysis system (SPACPASS) was constructed. The system is capable of first automatically assessing the collection efficiencies of the purification system and, second, accurately determining the purities of the collected fractions without the need for operator intervention. The double check device (DCD) proportionally samples a representative fraction from the purification flow stream only for the duration of the fraction collection event and enables automated post-purification analysis using an analytical HPLC column and the same chromatographic detectors. This report shows that the purity results obtained with the DCD and the SPACPASS agree with those acquired when sampling from a fractions that were evaporated and redissolved, one of the more traditional methods of post-purification fraction analysis.

2. Experimental

The SPACPASS was constructed starting with a standard Prep LC/MS system, a system that was proven reliable [23] and readily customizable [16,24]. The details of the operation of the commercial Prep LC/MS system have been clearly described elsewhere [23] The system used for this work consisted of the following commercial components: a Gilson 215 autosampler (Gilson, Wisconsin), an 819 injector (Gilson), three LC-8A Shimadzu pumps (Cole Scientific Inc., Moorpark, CA), a Shimadzu SCL-10A system controller (Cole Scientific Inc.), a Shimadzu SPD-10Avp UV detector (Cole Scientific Inc.), an API-150EX mass spectrometer (Applied Biosystems, Foster City, CA), an Alltech 500 evaporative light-scattering detector (ELSD) (Alltech and Associates, Deerfield, Illinois), a FC204 fraction collector (Gilson), a 10-port, two-position valve (Rheodyne labpro PR700-102-01) and a six-port, two-position valve (VICI, EHMA). Data was acquired using MassChrom 1.2.1, running on a Power Macintosh G4 (OS 9.0.4). FC Script 2.0 was used to control fraction collection. Three HPLC columns were used with this system: a semi-preparative column, Ultro 120 C18 U1-5C18-ME,

5 μm, 50 mm × 10 mm (Peeke Scientific, Redwood City, CA) and two analytical columns: C18-Q 5 μm, 50 mm × 4.6 mm (Peeke Scientific) and C18, 5 μm, 50 mm × 4.6 mm (Peeke Scientific). Eluents A–C were 0.05% trifluroacetic acid in water, 0.035% trifluroacetic acid in acetonitrile, and 0.049% trifluroacetic acid in 90/10 water/acetonitrile, respectively.

2.1. Switching valves and the double-check device

The heart of the system is the series of switching valves, A–C in Fig. 1. Switching valves A and B in Fig. 1, a 10-port, two-position valve and a six-port two-position valve, respectively accommodate two flow paths: the one used during preparative LC/MS and the other for analytical LC/MS analysis of the aliquot sampled 'on-the-fly' from the collected fraction. This configuration permits one of the columns to be re-equilibrated while the other is used for gradient elution.

The second six-port, two-position valve (Fig. 1, valve C) is used to store the representative aliquot sampled during the complete fraction collection event.

A custom application was written on a separate microprocessor that permits sampling and transient storage of this proportional aliquot collected from the flow stream for the duration of the fraction collection event. This representative sampling and storage was achieved by using a microprocessor controller to monitor the signal sent to the Gilson 204 switching valve from the FC 204 controller board and by triggering valve C, to collect from the flow stream while the FC 204 valve is triggered to collect a fraction. To allow fine control of aliquot collection, the controller allows for a user-selected delay of 0.0–1.0 s in 0.1 s increments.

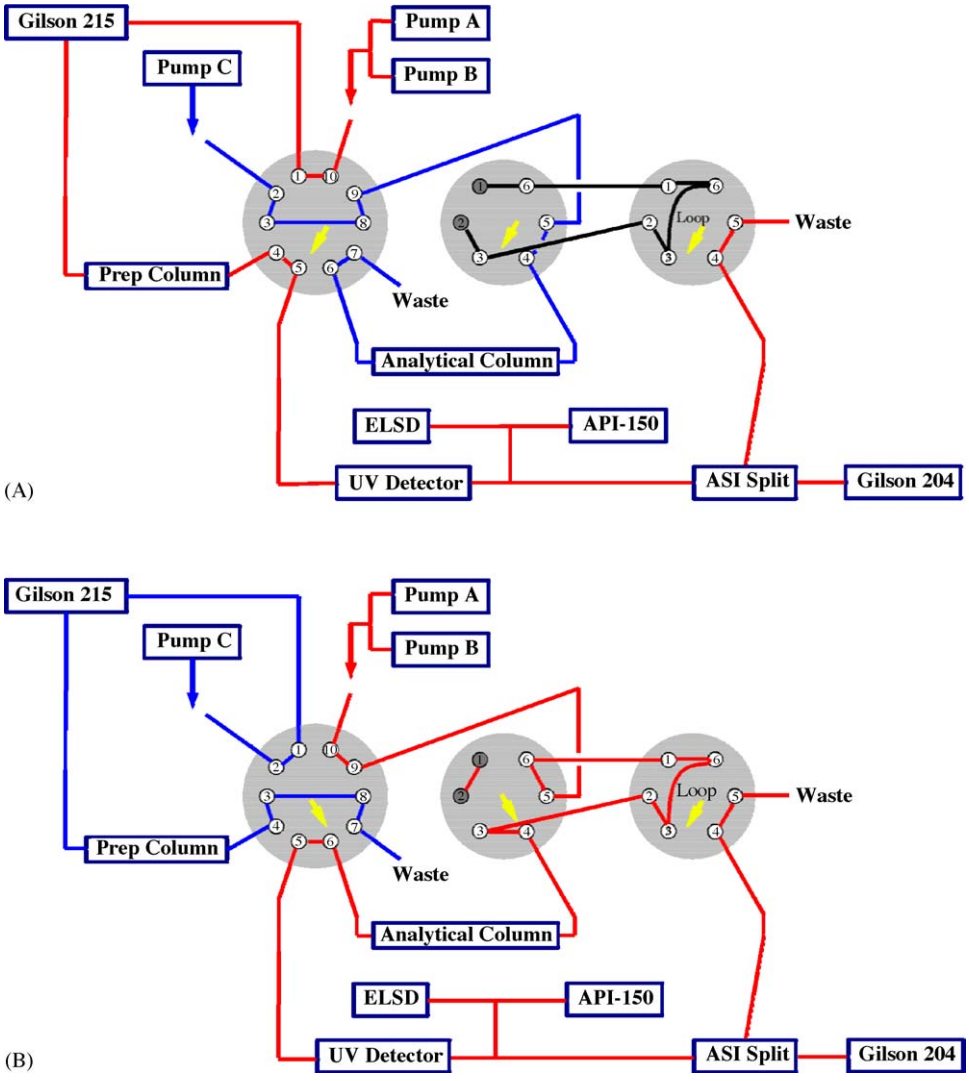

Fig. 1. (A) Prep mode: gradient running on preparative column. For the duration of fraction collection, the sampling loop, labeled 'Loop' on valve C, is switched in line with the effluent from the ASI splitter to collect a representative aliquot of the fraction collected on the Gilson 204 fraction collector. (B) Post-purification analysis of the aliquot sampled from the flow stream during fraction collection. The analysis is achieved by switching the sampling loop in line with the analytical flow stream. Once the sample is loaded, the loop is switched out to remove the excess volume.

2.2. HPLC pump program for Prep-QC

In order to test the feasibility of an in-line system for sampling a representative aliquot of the fraction collected, the LC/MS methods for purification and for post-purification analysis were combined into a single 9.2 min LC/MS method. During the first 5.5 min of the run, purification is done using the following generic conditions: 0.3 min load at 10% B, a 10% B–90% B linear gradient over 4.8 min, followed by a 0.3 min hold at 90% B and then a re-equilibration at 10% B. After 5.5 min, the system switches to begin the analysis of the aliquot collected by and stored in the DCD. The analytical LC/MS portion of the run consisted of: 0.5 min load at 10% B, 10% B–90% B linear gradient in 3.0 min, followed by a 0.25 min hold at 90% B. Aside from the drop in flow rates during the switching valve events at 5.5 and 9.0 min, the gradient flow rate remained at 6.0 mL/min during both the preparative and analytical portions of the run and the flow rate of the re-equilibration pump, pump C, remained at 4.0 mL/min. Thus, the combined total flow for the pumps was 10.0 mL/min.

With 0.20 mL/min of the total 6.0 mL/min gradient flow split evenly between the ELSD and the MS detectors, the 5.8 mL/min remaining flow rate is directed to the Gilson FC 204 fraction collector. Immediately before the FC 204 collection valve is a 20:1 splitter that diverts 0.3 mL/min from the total flow to switching valve C. Using a custom controller, valve C is synchronized with the fraction collector's switching valve so that it switches whenever the Gilson 204 fraction collector activates its switching valve, sending DCD to collection mode. Once the fraction collector stops collecting a fraction and diverts the flow to waste, the DCD also diverts the 0.3 mL/min flow to valve C to waste. By careful timing of the liquid flow paths, it was found that the liquid front of a collected peak arrived at the two valves within 0.1 s of each other. The proportional aliquot collected at valve C is directed into a 0.50 mL sample loop for temporary storage until its consumption during the analytical portion of the run.

2.3. Analytical LC/MS portion of Prep-QC

After the preparative portion of the run, DCD will switch into QC injection mode. Switching valve C places the 0.50 mL aliquot collection loop in line with the QC column for 30 s (3 mL), following which the DCD goes into QC mode by switching valves B and C in order to eliminate the dwell volume associated with the 0.50 mL loop, and the analytical gradient begins. This is shown in Fig. 1B.

2.4. Experimental design to validate novel proportional flow sampling device for post-purification LC/MS analysis

In order to determine if the aliquot collected using the in-line flow stream sampling device was representative of the collected fraction, calibration curves were constructed based on the aliquot sampled during purification and compared with those constructed after evaporation and reconstitution of the sample in 0.500 mL DMSO. If the novel collection device functioned as expected and sampled proportionately from the flow stream and obtained a representative sample, the Prep-QC results should be in agreement with the more traditional post-purification QC results. To examine this, stock solutions of 20 mg/mL Fmoc-Thr(tBu)-OH (Thr) and 10 mg/mL Fmoc-Ala-OH (Ala) were prepared. Samples were made to a total of 0.500 mL with 0.080 mL of Ala solution, DMSO and varying volumes of Thr solution to create Thr solutions spanning the range of 0.4–14 mg/mL, which is representative of the concentration range typically purified using this 50 mm × 10 mm I.D. HPLC column.

3. Results and discussion

In order to unambiguously ascertain the identity and purity of a compound, detailed analyses are done and generally include NMR [25] LC/UV(/MS), IR and chemical analysis [26,27]. Although such careful analyses provide valuable information, the investment required to apply this level of interrogation to all compounds synthesized may impact the productivity of chemists working in the discovery phase of a project. A more typical approach consists of comprehensive characterization of a few representative samples within a chemotype and LC/MS analysis for the others. In the lead development stage of a project, many compounds are synthesized, often around a small number of chemotypes. Thus, incorporating post-purification fraction analysis into the process of compound isolation reduces the time between synthesis and screening. In order to assess the applicability of the new technique, a comparison was made between the results obtained using this in-line sample and analysis technique and the results obtained using more traditional post-purification analysis.

With the DCD sampling 5% of the flow stream prior to the fraction collector switching valve, in order to preserve

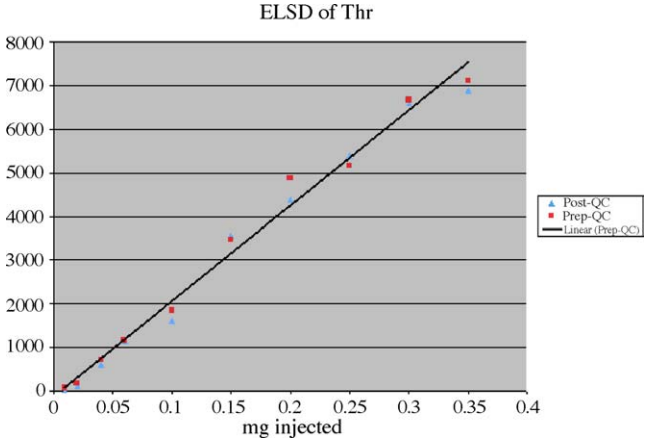

Fig. 2. Overlay of ELSD signal from during purification and post purification analyses.

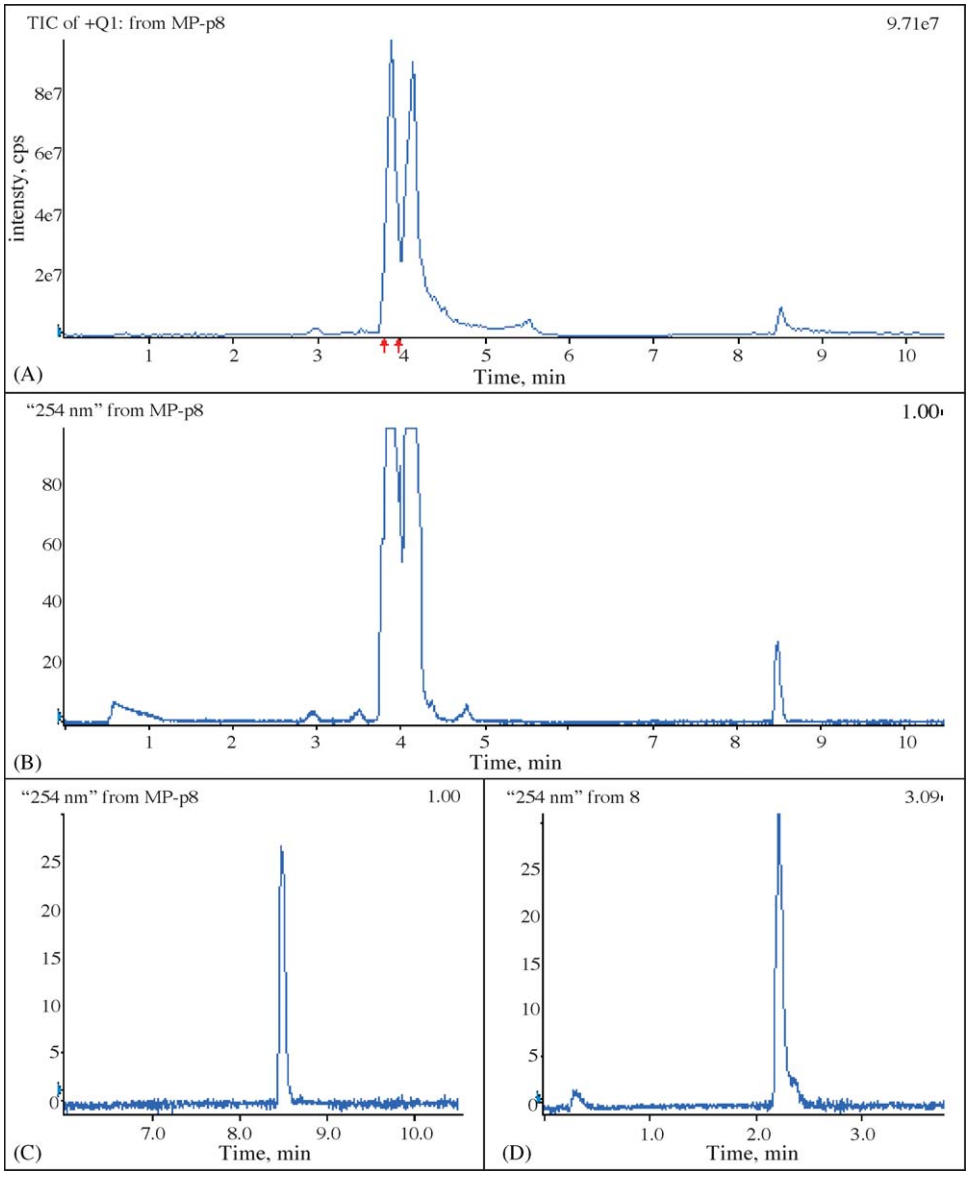

Fig. 3. Panes A and B contain preparative chromatographic data for a pair of closely eluting compounds. Panes C and D provide a comparison of chromatographic data acquired by immediately analyzing the sampled aliquot to that obtained via more conventional post-purification sample handling, respectively. Note that Pane C is an expanded view of the preparative chromatogram in pane B.

consistency, 5% of the final volume of each of the evaporated and redissolved samples was analyzed by analytical LC/MS. Fig. 2 is an overlaid plot of these two sets of data and indicates that the results obtained using the DCD and those obtained using the traditional post-purification LC/MS analysis are within the uncertainty reported for quantification by ELSD [28]. Since the results indicated that the sampling with the DCD is representative of what is collected, this suggests that post-purification analysis can be done immediately following the purification of each sample, thereby permitting the linking the purification and post-purification sample data within the same file. A typical chromatogram is shown in Fig. 3. Panes A and B are the total ion and UV 254 chromatograms for the preparative separation and for the aliquot analysis using SPACPASS. In this example, the small peak eluting at 8.5 min from the aliquot sampled from the flow stream. Fig. 3C is an expanded view of the post-purification LC/UV 254 chromatogram, and Fig. 3D is the post-purification analytical LC/UV254 data acquired after evaporating and redissolving the collected fraction.

Based on these results, selected compounds were purified and analyzed using the Prep-QC method described in this work and the results were compared with the analytical LC/MS results obtained following evaporation and redissolution of the sample in DMSO. The two methods of post-purification analysis provided comparable relative purity determinations (within ±5%) for >97% of the compounds collected. The chromatograms are similar *even though the*

DCD contains a 0.50 *mL loop that is used as the injection loop for the analytical portion of the Prep-QC method*. The aliquot collected in the loop does not form a homogeneous solution with the remaining non-displaced 10% acetonitrile solution in the DCD loop, and dispersion of the aliquot is further minimized because the aliquot leaves the sample loop for analysis through the same port into which is was introduced.

While there often appears to be a good correlation between relative purities measured before and after evaporation, some purified compounds may degrade on heating in a centrifugal evaporator, thereby removing the correlation between the pre-evaporation QC data and the post-evaporation sample. However, the same argument can be made that the data acquired for the post-evaporation sample may not reflect the purity of the compound that is assayed after sitting for one or more months on a HT-screening system [29]. Thus, for compounds stored at room temperature for HTS, it seems that reanalysis may be necessary to confirm the presence and purity of the expected compound once biological activity is confirmed. Thus, SPACPASS estimates the relative purity of the collected fraction and may serve as the reference for the post-screening analytical results.

There are a few limitations to the technique reported. Compatible solvent systems are required for post-purification purity assessment using the aliquot sampled from the flow stream during collection, thereby limiting somewhat the selection of stationary and/or mobile phases. In order to examine the accuracy of the DCD and the system's feasibility as a proof of concept, this work used similar columns for purification and analysis. In its current form it is applicable to the single pass purification and analysis of single fractions, so-called 1:1 collections, or for those processes that automatically recombine all collected fractions from a single purification. Both of these techniques have been used successfully for the purification of combinatorial [16] and focused [30] libraries. Although the system described would reduce instrument idle time, such serial processing does not provide as large an efficiency improvement as serial (or parallel) fraction collection and parallel analysis. Clearly the application of this technique could be extended by allowing for the sampling and analysis of aliquots from multiple fractions, particularly if these multiple aliquots could be analyzed using parallel chromatography [31]. Since the SPACPASS currently performs the post-purification analysis of the sample stored in the 0.500 mL loop of the DCD, regardless of whether a sample was collected or not, the system reported may not provide any reduction in the amount of LC/MS usage. In order to overcome this shortcoming, the system should be expanded to permit the collection of multiple fractions and the parallel analysis of aliquots sampled from collected fractions.

4. Conclusions

This paper reports a novel method that may be used to eliminate the traditional methods of post-purification analysis of collected fractions and to improve the efficiency of the overall purification process. The DCD was designed to sample representative aliquots from the flow stream prior to the fraction collection switching valve. The collection of these representative aliquots is achieved by intimately linking the sampling process with the fraction collection process so that the DCD collects for the entire duration of each of the fraction collection events. By comparing quantification data acquired using the DCD with data acquired using the more typical method of evaporation and redissolution prior to sampling, the results were found to be within the experimental uncertainty associated with ELSD quantification. A comparison of the relative purities of the collected fractions determined using the DCD and the more typical method of evaporating and redissolving samples prior to LC/MS analysis indicate that the two methods provide the same results. Based on the work outlined in this report, this technology is being used to streamline the purification and post-purification analysis of small focused libraries produced by the automated synthesis group.

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