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Automated approach for the rapid identification of purification conditions using a unified, walk-up high performance liquid chromatography/supercritical fluid chromatography/mass spectrometry screening system

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ABSTRACT

Integration of supercritical fluid/mass spectrometry (SFC/MS) and reversed phase liquid (HPLC/MS) chromatographic screening techniques into a single chromatographic system and utilized in "walk up" mode, enabled us to produce an orthogonal data set for selecting purification conditions for medicinal chemistry compounds. To streamline the overall workflow, we also demonstrate the use of automated batch data processing of individual data files to identify suitable separation conditions without user intervention. We have addressed the chromatographic challenges that hinder the identification of the intended target and thus the selection of ideal purification conditions. For instance, multiple component-of-interest (COI) peaks, co-elution of impurities with the COI, and chromatographic suitability factors such as retention times and peak shapes are all important considerations when selecting appropriate methods for purification and, therefore, are bottlenecks to an automated approach. Since SFC and HPLC data were collected in parallel from separate instruments in our workflow, the time required for the separation scientist to analyze acquired data from both systems was a time-limiting factor. To reduce data processing time and accelerate or "FastTrack" samples to purification, two unique and automated solutions were introduced. We describe the implementation of an integrated, multi-column, walk-up HPLC/SFC/MS system, and the implementation of an intelligent, automated method selection application which uses advanced data evaluation criteria to selectively score and identify the most practical separation conditions for SFC/MS and HPLC/MS methodologies.

1. Introduction

Multi-column analytical screening is a common approach for accelerating method development for chromatographic purifications [1–7]. At Pfizer, both SFC and HPLC are used to enable chromatographers to maximize successful purification outcomes. The orthogonality of HPLC and SFC provides separation scientists with an increased chance of obtaining suitable separation conditions for the purification of medicinal chemistry lead compounds [8–11]. However, due to the larger number of separation profiles generated from both HPLC and SFC data, the process of analyzing, comparing and contrasting each piece of acquired data and selecting the optimal purification conditions can be time-consuming. For instance, just five samples undergoing screening against six columns will produce a total of 30 individual sets of data. Assuming each dataset requires an average of 30 s to review and interpret, the decision-making process for 5 samples could take approximately 15 min or longer. Data analysis is further complicated by the presence of multiple target or multiple component-of-interest (COI) peaks within an analytical screening run, which hinder the identification of the intended target; co-elution and incomplete resolution of target and impurities; and chromatographic suitability factors such as retention time and peak shape. All of these are important considerations when selecting the appropriate method for purification.

In order to improve our purification workflow efficiency, we apply automation wherever feasible. This is especially important when the sheer number of compounds being analyzed and purified can lead to excessive turnaround times [12]. In order to maintain any reasonable efficiency, we have either developed or adopted automated HPLC or SFC screening approaches, sacrificing optimal conditions and batching samples for analysis. In a lower throughput environment, more methods can be employed in the screen but the majority of the data evaluation is still performed manually by the separation scientists themselves. However, we found that batching of samples actually led to slower throughput as these

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unrelated samples waited for the entire batch to run, instead of processing them individually.

Medicinal chemists have long depended on open access HPLC/MS systems for reaction monitoring, mass confirmation and/or relative purity (% area) assessments, because these user-friendly systems produce rapid and reasonable results [13–16]. Over the past 10 years SFC has been recognized as a valuable chromatographic tool for separation scientists, but the absence of user-friendly SFC instrument and associated software has limited its use to chromatography experts. The development of SFC technology that converts an existing HPLC system to an SFC has paved the way for SFC to gain acceptance as a more mainstream self-service technique for medicinal chemists. We took advantage of this system to implement the "FastTrack" approach to accelerate target chemistry lead purification by having the medicinal chemists initiate the screening themselves, thus allowing the chromatographers to focus more on purification-related tasks [17]. To further enhance the separation capabilities while reducing the capital and space footprint of additional walk-up systems for the "FastTrack" screening, we combined HPLC/MS and SFC/MS into a single system as reported in detail here.

While this system simplifies the sample submission process for the end-user, it does not address the time restraints associated with the data analysis and method selection of the screening data. Several groups have utilized different software applications for automated batch data processing, but the method selection process is still a limitation to a fully automated approach. Zeng et al. developed customized intelligent software for selecting appropriate separation methods for enantiomers based on the resolution of the enantiomer peak pair [18]. Weller et al. implemented an automated method selection software which identifies the nearest peaks immediately before/after the target peak and determines the resolution from the target peak [19]. However, with orthogonal techniques, impurity retention may vary dramatically between methods and lead to hidden, unresolved and partially resolved peaks co-eluting with the target. We solved this by aggregating ions into two groups, the COI and the unknown ions, which then enabled us to account for all possible impurities as they shifted retention from method to method without specifically identifying each impurity.

To further improve the efficiency of the purification workflow and remove the complexity and time restraints associated with data analysis, an intelligent, automated method selection package was implemented that enables automated data processing, selective scoring, and visualization of acquired SFC/MS and LC/MS data. The software then selects the most appropriate methods using customized scoring algorithms. Samples that are submitted to the analytical LC/SFC/MS screen are processed to automatically select the method with the highest method score as the "best" separation method to use. This combination of walk-up integrated LC/SFC/MS system and intelligent method selection provides us with a nearly fully-automated solution for improving overall workflow efficiency.

2. Experimental

2.1. Chemicals and materials

Commercially available Ultra LC/MS™ grade methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, USA); OmniSolv high purity water from EMD Chemicals, Inc. (Gibbstown, NJ, USA); and ammonium acetate 99.99+%, trifluoroacetic acid 99+% spectrophotometric grade, caffeine, theobromine and etofylline from Sigma–Aldrich (St. Louis, MO, USA). Carbon dioxide (CO_2) and nitrogen (N_2) are bulk grade and purchased from AirGas West

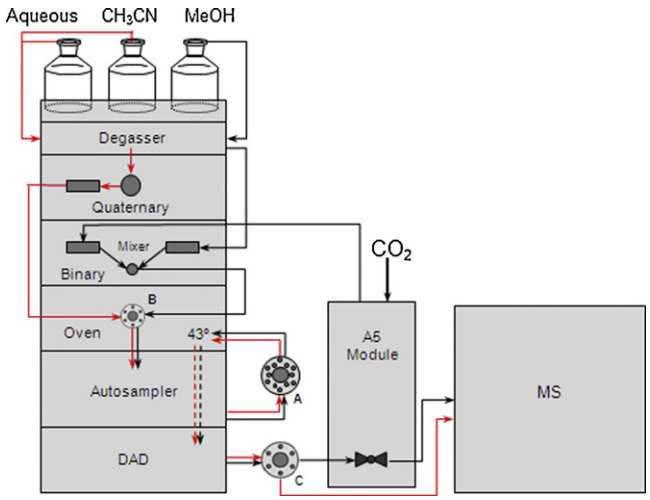

Fig. 1. Diagram of the integrated HPLC/SFC/MSD system. The red lines indicate the flow path for the HPLC solvents using the quaternary pump. The black lines indicate the flow path for the SFC solvent using the binary pump. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Escondido, CA, USA). The CO_2 supplied to this system was purified and pressurized to 1500 psig using a custom booster and purifier system from Va-Tran Systems, Inc. (Chula Vista, CA). Stock solutions of caffeine and theobromine were prepared to a concentration of 500 μg/mL in methanol, and etofylline to 750 μg/mL, and a mixture was prepared from 250 μL aliquots of each. Pfizer proprietary compounds were also used for the purposes of this paper, and screening samples were prepared in methanol to a target concentration of approximately 0.2 mg/mL.

2.2. HPLC/SFC/MS configuration

All chromatographic analyses were carried out on a combined HPLC/SFC/MS system, which consisted of Agilent 1100 Series HPLC (Palo Alto, CA, USA) components including a G1322A degasser, G1312A binary pump for SFC solvents, G1311A quaternary pump for HPLC solvents, G1313A autosampler (ALS), G1316A column compartment with an internal 2-position, 6-port valve, and a G1315B diode array detector outfitted with a 10 mm, 13 μL high pressure flow cell (400 bar). This HPLC system was converted to use as a supercritical fluid chromatograph by integrating it with an Aurora SFC Fusion™ A5 (Aurora SFC Systems, Inc., Sunnyvale, CA, USA) module and through modifications made to the ALS and binary pump as described elsewhere [20]. The system was interfaced to an Agilent 1100 Series G1946D MSD single quadropole mass spectrometer equipped with an Atmospheric Pressure Chemical Ionization (APCI) source. Since the column switching valve was external to the column oven for this system, the SFC and HPLC columns were considered to be at ambient temperature. To minimize baseline noise caused by thermal differences between the mobile phase leaving the column and entering the DAD for SFC mode, the right heat exchanger in the column compartment is used to preheat the effluent to 43 °C [21].

The system also utilizes an Agilent 1200 series G1159A 6-column selection valve (Valve A) for the SFC and HPLC columns, and a 1200 series, G1158A 2-position, 6-port valve (Valve B) to bypass the Aurora Fusion A5. The transfer line between Valve B and the APCI source is a 75 cm length of 0.005 in. PEEKsil tubing (Upchurch Scientific, Inc., Oak Harbor, WA, USA). Fig. 1 illustrates the configuration of the integrated LC/SFC/MS system. All SFC columns were purchased from Zymor, Inc. (Wayne, NJ, USA), and the HPLC columns were purchased from Phenomenex, Inc.

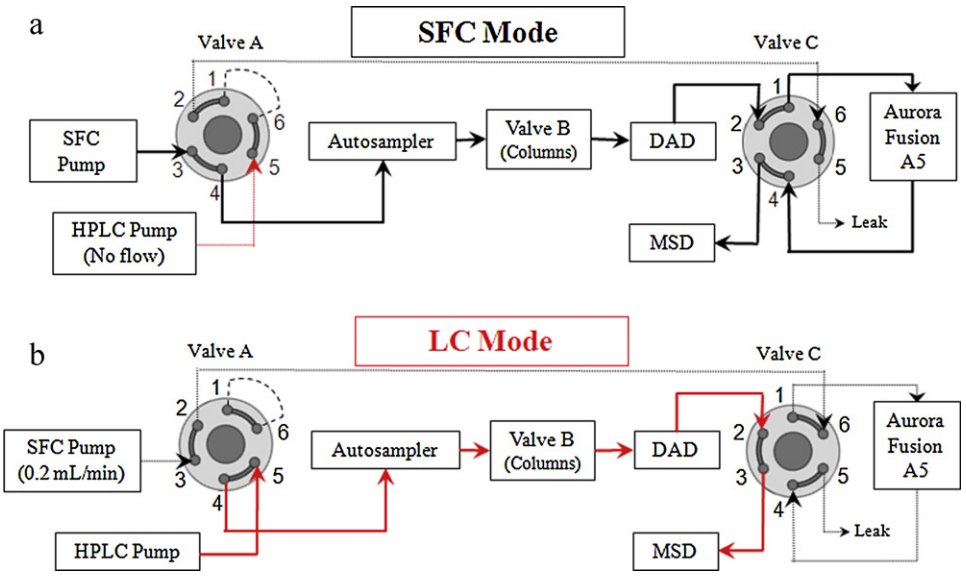

Fig. 2. Schematic of the mobile phase flow paths in (a) SFC mode of operation and (b) HPLC mode.

(Torrance, CA, USA). SFC column dimensions were 150 × 4.6 mm i.d. with 5 μm particles and 100 Å pore sizes, while the HPLC columns were 50 × 4.6 mm i.d. with 5 μm particles and 110 Å pore sizes.

2.3. SFC/MS and LC/MS flow paths of integrated system

In order to successfully integrate both reversed phase HPLC and SFC capabilities into a single system, it was necessary to incorporate three multi-position valves which serve to alternate between SFC and HPLC flow paths. The flow paths for both the SFC and HPLC modes of operation are presented in Fig. 2. Valve A, which is located internally within the column compartment, is a 2-position, 6-port valve connected to both the HPLC and SFC pumps. Valve B, is a 6-position column switching valve which houses the six stationary phases for the LC/SFC/MS screen. Valve C, is a 2-positon, 6-port valve which serves to bypass the A5 module during HPLC runs and, as a result, minimizes potential maintenance issues from exposure to the HPLC mobile phase and eliminates extra dead volume from the system. Both Valves A and B are external to the LC/SFC/MS system, and all three are controlled by the methods in ChemStation. In SFC mode, Valve A is positioned such that the mixed CO_2/methanol mobile phase passes through ports 3 and 4 to the autosampler, as shown in Fig. 2a. There is no flow from the LC pump, which is connected to port 5. Valve B is positioned so that SFC flow is directed to one of the four SFC columns. After passing through the DAD, the effluent continues through ports 1 and 2 of Valve C to the A5 module back pressure regulator (BPR), and finally to the MSD via ports 3 and 4 of Valve C. When the HPLC run is initiated, the valves switch to enable the flow of HPLC mobile phases through the system.

Several factors were found to affect normal operation of the A5 module as a result of the added complexity of the combined system. While switching between SFC and HPLC modes, the system pressure for SFC must be maintained. Setting the SFC flow to 0 mL/min during data acquisition in HPLC mode resulted in several failure cascades of the booster pump. Without delivery of CO_2 flow to the A5, the system attempts to equilibrate, during which the sample sequence is paused. If the pressure stabilization time for the A5 is exceeded, the system shutdown events are triggered. To enable timely pressurization and stabilization of the booster pump before the shutdown occurs, it was necessary to implement a workaround to the SFC flow path. In Fig. 2b, a 90 cm length of 0.17 mm i.d. stainless steel tubing was connected from Valve A in the column oven to port 6 on Valve C. The SFC pump was then set to deliver 100% of CO_2 to the A5 module at a flow rate of 0.2 mL/min in parallel with the flow of the HPLC pumping system. This SFC flow was just enough to keep the system fully pressurized and enabled the A5 BPR to maintain system pressure under HPLC conditions. To prevent inlet overpressure at the BPR, a small, isolated length of tubing, acting as a "leak" at Valve C was introduced at port 5. Different lengths and internal diameters of tubing for the "leak" were evaluated and system stabilization time was determined for each. A stabilization time of less than 1.5 min was achieved with no back pressure fluctuations when switching from SFC to HPLC modes by using a 60 cm length of 0.17 mm i.d. stainless steel tubing.

2.4. HPLC/SFC/MS screening conditions

Each submitted sample goes through an automated six-column screen which includes four SFC and two HPLC columns. Table 1 lists the stationary phases used for the screen and their mobile phase conditions. All samples are screened using the SFC columns first before proceeding to the HPLC analyses. The injection volume is 15 μL. For the SFC portion of the screen (methods A1–A4), no acidic or basic additives were used in the mobile phase to attempt the isolation of the free base product upon purification. The flow rate for SFC mode was 3.5 mL/min, and the column outlet pressure was maintained at 140 bar. Total cycle time for each run was 5.0 min. While the system is in SFC mode, the HPLC quaternary pump flow rate was set to 0 mL/min. Since the overall retention on the HA-DP and HA-P stationary phases appears to be higher for the compounds tested to date, the starting modifier percentage was raised from 5.0% to 7.5% for those two columns in order to maintain a consistent throughput. This change did not have an adverse effect on peak shape or selectivity.

For the HPLC runs, two separate screening methods (B1–B2) were used. Both employed the same relative gradient conditions but with different additives in the aqueous phase. The differences in pH of the TFA (~1.5) and ammonium acetate (~5.5) buffered mobile phases accounted for the range of diverse compounds being screened. The mobile phase flow rate was maintained at 2.25 mL/min, and the cycle time was 4.50 min. After the completion of HPLC method B2, the system reverts back to SFC mode. If no new samples are immediately available, the system will go into standby (SFC pump flow rate will drop to 0.2 mL/min. and 100%

Table 1
Stationary phases and conditions used for the HPLC/SFC/MS screening.

Method	Column	Mobile phase	Gradient conditions
A1	ZymorSPHER Pyr/Diol (pyridine and diol mixed phase)	1-CO_2 2-Methanol	5–50% (2) in 3.4 min; hold for 0.6 min
A2	ZymorSPHER HADP (HA-dipyridinyl)	1-CO_2 2-Methanol	7.5–50% (2) in 3.4 min; hold for 0.6 min
A3	ZymorSPHER HAP (HA-pyridinyl)	1-CO_2 2-Methanol	7.5–50% (2) in 3.4 min; hold for 0.6 min
A4	ZymorSPHER DIOL/MONOL (diol and monol mixed phase)	1-CO_2 2-Methanol	5–50% (2) in 3.4 min; hold for 0.6 min
B1	Phenomenex Gemini C18	1-Acetonitrile 2–10 mM ammonium acetate in H_2O	0–100% (1) in 3.0 min; hold for 0.75 min
B2	Phenomenex Gemini C18	1-Acetonitrile 2–0.05% TFA in H_2O	0–100% (1) in 3.0 min; hold for 0.75 min

CO_2) until a new sample sequence is initiated. Therefore, the SFC methods always precede the HPLC runs on the unified HPLC/SFC/MS system.

UV detection was monitored at wavelengths of 220 nm and 260 nm, with the slit width set to 8 nm and the peak width set to >0.05 min (1 s response time). Product peak identification was aided by positive ionization mode APCI, with the following MSD spray chamber settings: dry gas flow and temperature were set to 12 L/min and 350 °C, respectively; nebulizer pressure 50 psi; vaporizer temperature 450 °C; capillary voltage 3000 V, and corona current 4.0 μA.

2.5. Sample submission and data analysis software

All data was acquired using Agilent 32-bit ChemStation™ (Version B.03.01 [317]), which enables control of all the hardware system components. Agilent Easy-Access (Version A.5.01 [Build 290]) is a user interface software that was used to solicit the necessary sample information from the user, instruct the user to load the samples onto the autosampler, and begin the column screen. Samples are submitted to the HPLC/SFC/MS screening workflow through the Easy-Access interface as shown in Fig. 3. Each sample is identified by a 5-digit barcode number. The screening is then initiated by the submitter, and each sample goes through the six-column screening protocol. Due to the six-method per sample restriction imposed by Easy-Access, we chose to use four SFC and two HPLC methods for the screen.

The intelligent software package used to automate the data processing and select the best chromatographic methods consisted of Analytical Studio Express Server (AS-Express) and Analytical Studio Professional (AS-Pro), both running the Compound QC Workflow (Virscidian, Inc., Raleigh, NC, USA). Data evaluation criteria and visualization of processed LC/SFC/MS data was achieved using a combination of AS-Pro functions and customized scoring expressions.

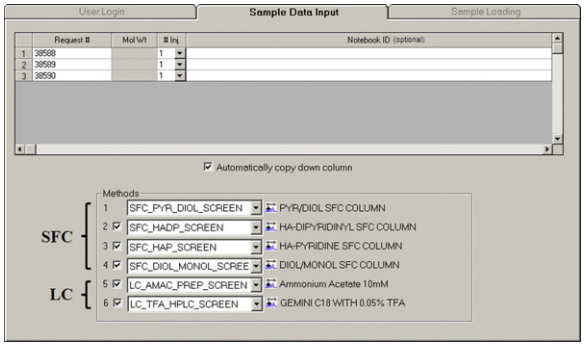

Fig. 3. Example of the user interface where sample information is entered prior to initiating the screening sequence.

3. Results and discussion

3.1. Screening workflow

The accessibility of a walk-up SFC system allowed medicinal chemists (submitter) to initiate the sample screening process when they submit their samples for purification [17]. Routine sample screening and method development time by the chromatographer were minimized which enabled them to concentrate on data processing and review tasks. Generation of acquired data commences as soon as the sample screening is initiated by the submitter. Therefore, batching of samples as well as long wait times for sample data was eliminated. Raw data files are then available for review by the chromatographer upon completion of the screening protocol for each sample. This serves to expedite ("FastTrack") samples to the SFC purification workflow. However, manual review of each piece of screening data was required in order to assign the best chromatographic parameters to use for purification. This manual workflow is outlined in Fig. 4a.

In cases where purification is not amenable to SFC, HPLC screening performed on a separate system further contributed to the data analysis time and added to the complexity and inefficiency of the screening process. Implementation of the integrated HPLC/SFC/MS system for the screening of compounds for purification is only the first step towards improving overall purification workflow efficiency. To achieve a condensed, more streamlined workflow as shown in Fig. 4b, automation of the data acquisition and method selection process was added to remove the complexity and time restraints associated with data analysis.

3.2. Customized method selection & visualization

Separation scientists often encounter screening data that requires more detailed and timely analysis to determine method suitability for purification. Multiple component-of-interest (COI) peaks within a single run, as well as co-elution and incomplete resolution of target and impurities, can hinder the identification of the intended target. Additionally, chromatographic suitability factors such as retention times and peak shapes also need to be considered. All of these are important factors which influence the selection of appropriate purification methods for each sample and, therefore, are bottlenecks to an automated workflow. To overcome these challenges, a unique software application was implemented which automates the analysis, review, and selection of ideal HPLC and SFC purification conditions from the HPLC/SFC/MS screening data.

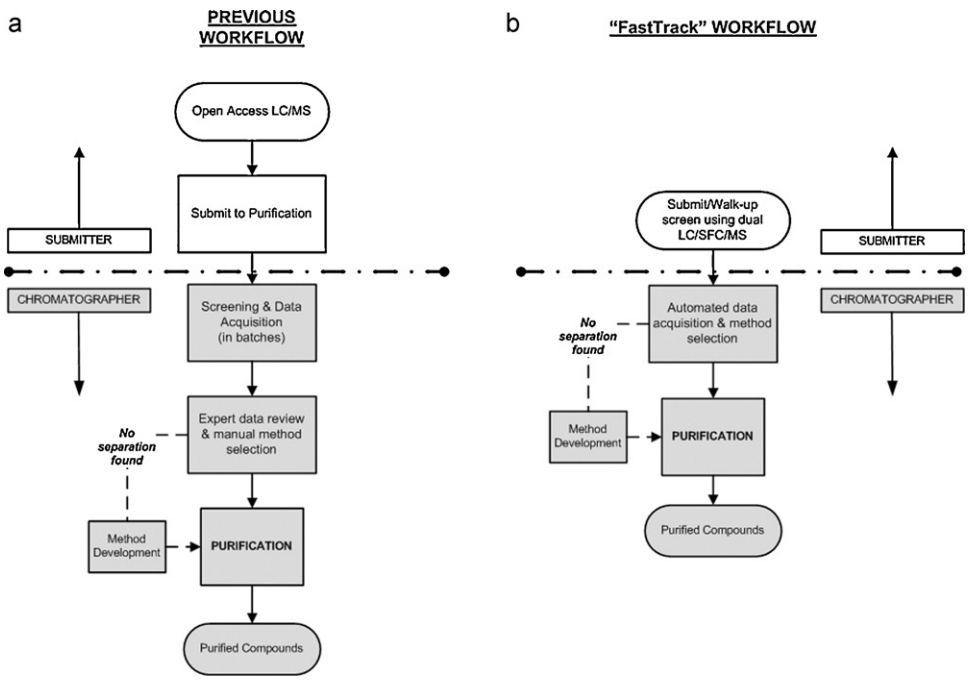

Fig. 4. Flowcharts highlighting the reduction in steps in the overall workflow. (a) The previous workflow required manual data analysis following sample screening, while (b) the "FastTrack" workflow facilitates direct-to-purification through automated data processing and method selection.

3.2.1. *Method selection expressions*

At the end of each run, the ChemStation-acquired data is uploaded to the AS-Express server where raw data undergoes automated processing against user-defined data processing conditions. After all six screening runs for a particular sample have been completed, a customized processing operation for method selection scoring is applied across all methods. The scoring expressions and their rating benchmarks are presented in Table 2. Since COI peak shape and resolution of its nearest neighbor(s) are important factors in scale-up, algorithms which took these expressions into account were applied as a tool to measure chromatographic suitability for purification. For instance, if there are multiple COI peaks in at least one run, the algorithm expects to find multiple COI peaks in every run and, thus, will score each run accordingly. If a single COI is found during a run for that sample, the assumption is that co-elution is present and, therefore, will be assigned a lower score.

Initial evaluations of approximately 75 sample screening results show that using the sum total of the scores to rank the methods is highly dependent on the accuracy of the calculated expressions. These scoring criteria are detailed in the next section. In cases where we questioned the selection of the "best" method, a refinement of the integration parameters eventually led to a correction in the method choice. Since each selection criteria is weighted due to

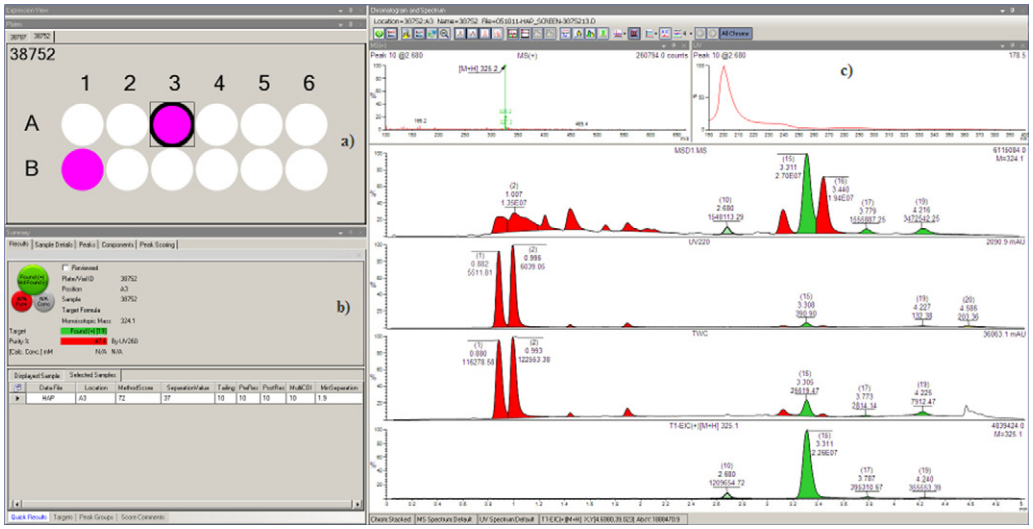

Fig. 5. Example of result visualizations for sample ID 38752 using method A3. (a) The virtual plate view highlights the "best conditions" by SFC (row A) and HPLC (row B) in pink. (b) Scoring result information for the sample using SFC method A3. (c) User-defined chromatogram and spectrum view, displaying (from top to bottom) mass spectra, UV220, TIC, TWC and EIC channels. The target COI peak is indicated in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Scoring expressions and specific criteria used for method selection.

Expression	Description	Scoring Criteria
Separation value	Determines degree of co-elution and separation of product and impurities. Calculates pre- and post- resolution between main peak in the composite COI chromatogram and the nearest unknown composite peak. The smallest of these values is the minimum resolution.	Minimum resolution < resolution threshold, value is 0; Minimum resolution > resolution threshold, value is scaled 0–100; Minimum resolution > maximum threshold, value 100; No unknown peaks observed, value 100
Minimum separation	Unscaled minimum resolution of the main composite EIC COI peak and its neighboring impurities. Value is then scaled to give the Separation Value score.	Not used for scoring purposes
Tailing	A measure of best COI peak shape. Value is based on the calculated tailing factor of the best COI peak in the TWC.	No COI, value is 0; Tailing factor = midpoint, value is 10; Minimum < tailing factor > maximum, value is 1; Minimum > tailing factor < maximum, value is scaled 1–10
Pre resolution	Resolution of neighboring peaks in the TWC[a]. TWC channel is used to match a UV-triggered purification approach, especially for SFC.	No COI, value is 0; Pre resolution < minimum, value is 0; Minimum > pre resolution < maximum, value is 5; Pre resolution > maximum, value is 10
Post resolution	Resolution of neighboring peaks in the TWC.	No COI, value is 0; Post resolution < minimum, value is 0; Minimum > post resolution < maximum, value is 5; Post resolution > maximum, value is 10
Multi COI Peaks	Determines presence of multiple peaks with the same *m*/*z*	First pass: no COI, value is 0, 1 COI, value is 10, >1 COI, value is 5; Second pass (across all samples): >1 COI, value is 10; others with 1 COI, value is 5

[a] TWC, total wavelength chromatogram.

its perceived importance in scale-up, optimization of these parameters is ongoing and further improvements are expected.

3.2.2. Results of processed data

When samples are added to the purification queue, the processed sample data is queried and downloaded into AS-Pro. A comprehensive visualization of the data for sample ID 38572 is presented in Fig. 5. In the virtual plate view, rows A and B represent the SFC and HPLC runs, respectively, while each circle or well represents a different screening method (refer to Table 1). The individual method expression scores for all six methods (Table 3) can be viewed by the chromatographer to rationalize the selected methods, and the pre-selected SFC and HPLC methods are highlighted. Among the six method scoring variables, the most significant is the separation value, which can be defined as a measure of the quality of the separation. It is a scaled factor based on the resolution between the COI peak and the next nearest impurity (unknown) peak from a reconstructed and overlaid EIC chromatogram of all contributing component masses of the COI and an those from a cumulative list of unknown masses (Fig. 6). To calculate the separation values, all mass-to-charge (*m*/*z*) ions found in each spectrum are tabulated separately for each method within a sample set. The *m*/*z* list generated from each method is then compared to the other methods to determine the presence of recurring ions. Ions that consistently fall within the COI region and have the same *m*/*z* ratio relative to the COI ion across all runs are placed in the composite COI group. These include isotopic, fragment and adduct ions. All other common ions found outside the COI region are moved to the composite unknown group. Based on the user-defined criteria for separation value, the resolution is scaled between the minimum and maximum criteria based on its position from 0 to 100. This enables the method score to be weighted appropriately for methods with good sample resolution.

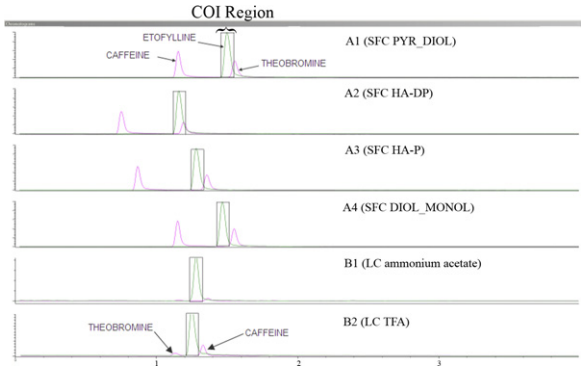

Fig. 6. Composite chromatograms of the separation of the etofylline COI (green trace) and caffeine and theobromine impurities (purple trace) across all six methods. Separation analysis is performed by comparing *m*/*z* found in the COI region of each method and measuring the degree of resolution of the nearest impurity peak, regardless of identity. For instance, the separation value from etofylline is determined using theobromine in methods A1-A4, but caffeine is used for methods B1-B2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

We assume that the probability of two separate compounds co-eluting across all six methods from two orthogonal chromatographic techniques is minimal. Once the aggregated ions are appropriately grouped, separate composite chromatograms are rendered, overlaid, and the resolution criteria are reapplied. This

Table 3
Scoring summary results for sample ID 38752.

Method name	Method score	Separation value	Tailing	Pre resolution	Post resolution	Multi COI	Minimum separation
B1 (LC AMAC)	130.000	100.000	8.000	7.000	10.000	10.000	3.700
A3 (SFC HA-P)	72.000	37.000	10.000	10.000	10.000	10.000	1.900
A1 (SFC PYR_DIOL)	58.000	32.000	1.000	10.000	10.000	10.000	1.800
A2 (SFC HA-DP)	32.000	0.000	7.000	10.000	10.000	10.000	0.000
A4 (SFC DIOL_MONOL)	32.000	0.000	7.000	10.000	10.000	10.000	0.000
B2 (LC TFA)	32.000	0.000	7.000	10.000	10.000	10.000	0.000

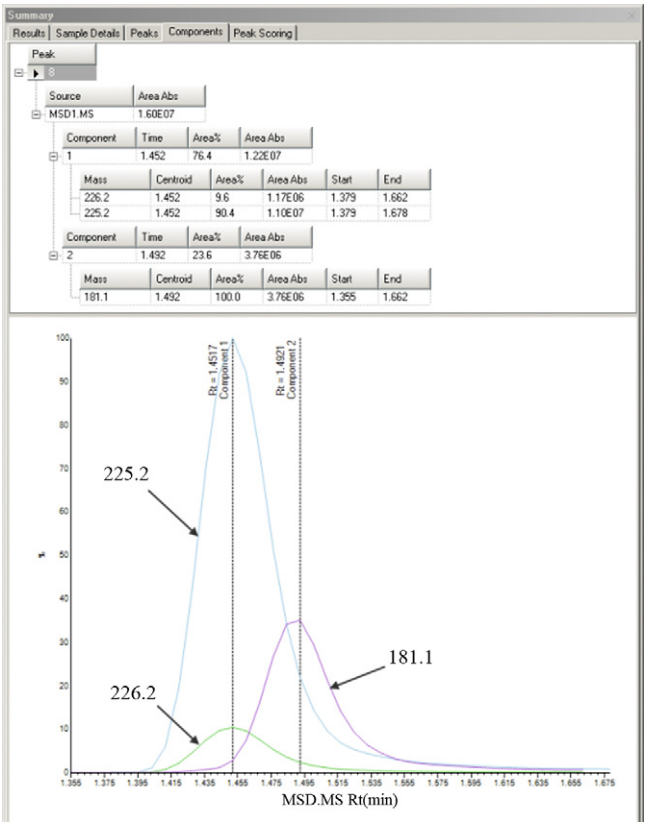

Fig. 7. Example of the componentized COI window clearly indicating the co-elution of theobromine (m/z = 181.1) with etofylline (m/z = 225.2) and its corresponding C^{13} isotope (m/z = 226.2).

forms the basis of a unique visualization tool called the separation view, which enables the chromatographer to quickly assess all the screening methods to validate the scoring results, or to offer the flexibility of utilizing another method (e.g. to batch several samples together using one purification method). Fig. 6 shows the separation view for a mixture of etofylline (COI), and caffeine and theobromine (unknowns) during a screening run. These reconstructed chromatograms represent the extracted ion (EIC) for COI, as well as those for all unknown ions that are found to elute in all six chromatograms. To illustrate the fundamental importance of the separation value, selectivity differences are clearly observed for each method, and the selected separations are the A4 (SFC DIOL_MONOL) and the B1 (LC ammonium acetate) methods. Separation values are calculated by considering the resolution between the COI and the nearest neighbor which, for the SFC runs, is theobromine. As an example, the A2 (SFC HA-DP) method demonstrates a situation where overlapping peaks (etofylline and theobromine) produce an inconsistent ratio of ions across the COI region, indicating the presence of a co-eluting impurity. While the composite EIC chromatograms are designed to highlight the presence of unknown ions relative to the COI, the componentization view in Fig. 7 is used to observe the other ions present as well as their abundances within the COI region. In this case, the co-elution of theobromine with etofylline and its corresponding C^{13} isotope is clearly observed. Since the elution order of theobromine and caffeine for the LC runs is reversed, the nearest neighbor calculations are based against caffeine. In this way, chromatographers will be able to assess any potential co-elution without specifically tracking any one impurity. Overall, utilization of the separation view enables the chromatographer to quickly and visually determine if co-elution occurs without specifically needing to track individual impurities. Therefore, compared to the other expressions, the separation value is probably the most useful quantitative measure for determining the "best" separation conditions for a sample.

The composite extracted chromatogram view is also beneficial where multiple COIs are present in a sample such as that presented earlier (sample ID 38752). In such cases, the main COI band, or reference COI, is used for scoring purposes. The separation view for the screening of sample ID 38752 is shown in Fig. 8, where multiple COI peaks are present in all methods, including the reference COI peak and one minor one. In addition, co-eluting unknowns with the COI peaks can be observed for all methods; however, the ratio of impurity peak area relative to the main COI peak area for the A1 (SFC PYR_DIOL), A3 (SFC HA-P) and B1(LC ammonium acetate)

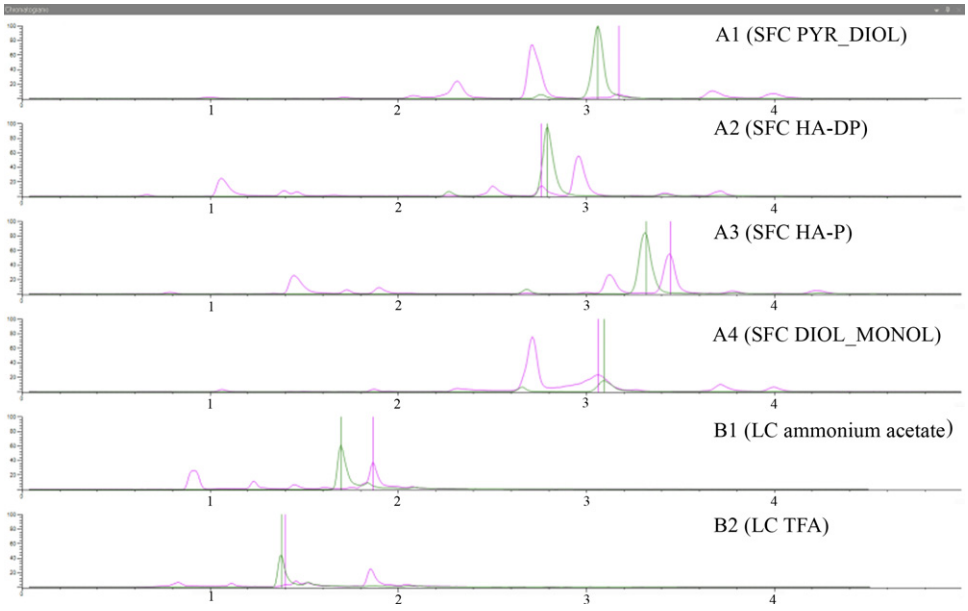

Fig. 8. Composite chromatograms of COI (green) and unknowns (pink) for sample 38752 with vertical lines indicating the COI and impurity peaks used for the separation analysis. This feature is particularly useful when multiple COI peaks are observed. The highest scoring methods for SFC and HPLC are A3 and B1, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

methods fell below a user-defined threshold, while the others did not. As a result, these three A1, A3 and B1methods received scaled separation values (Table 3) indicating a pure COI peak. COI peaks for the other methods were deemed impure under those conditions. Additionally, the A3 method demonstrates good overall peak shape and good resolution from its nearest neighbors; therefore, it was selected as "best" SFC method. B1 was selected as "best" HPLC method. Comparatively, the A1 method did not score as well in the expressions for peak shape and separation values, but upon visual inspection of the composites, appears to be a good secondary SFC candidate to the A3 method. Since the MS signal can sometimes distort peaks due to interface efficiency, we chose to determine these resolution values based on the total wavelength chromatogram (TWC) UV signal for purification trigger purposes. For the sample ID 38752, a MultiCOI score of 10 was given for all screening runs, which indicates the presence of only one COI peak in the MS-EIC channels.

The main reason for tracking the MultiCOI parameter is due to the potential for regioisomers and diastereomers, which result in multiple fractions in mass-directed purifications. This knowledge is meant to aid in the selection of the appropriate fraction(s). The MultiCOI score is calculated using two criteria, one used on the individual sample run and the other on the all the runs within a sample screen. The first algorithm determines the presence and number of the COI peaks. The second algorithm compares the number of COIs from each method to determine which method results in the best separation of COI peaks. In cases where there is only one COI found within the MS-EIC channel of each method, the MultiCOI score is 10 for all the runs. Additionally, screening runs with multiple COIs will have their MultiCOI scores reduced to 5. However, if more than one run within a sample screen contains multiCOIs, these receive a score of 10, while the runs with a single COI are downgraded to a score of 5 for that method, indicating a non-separation. All methods with no COI are scored zero.

The chromatographer can change the visualization to show the best score either across ALL samples irrespective to technique or as a function of each technique. If the latter is chosen as in this case, then AS-Express will select the best SFC method and the highest scoring HPLC method even if co-eluting impurities are present (null separation values). Therefore, the highest score achieved among the SFC runs is the A3(HA-pyridinyl column), and the highest HPLC score was obtained by B1 (ammonium acetate conditions). However, the actual technique used to purify each compound is left to the discretion of the chromatographer. Overall, these unique visualization tools for processed chromatographic data of the LC/SFC/MS screening runs minimizes data reviewing time and the decision-making process, and thus allows for a more efficient purification-focused workflow. Review of ten samples now takes less than 2–3 min, both decreasing the time required for reviewing data from each run, and increasing the overall sample throughput through this step in the process workflow.

4. Conclusion

We have succeeded in creating a simple, more streamlined workflow to accelerate or "FastTrack" samples to purification. Implementation of a unified, walk-up HPLC/SFC/MS system reduces the need to screen samples on two separate HPLC and SFC screening systems. It enables submitters to initiate the screening of their samples and eliminates the batching of samples prior to screening for faster data acquisition. The utilization of the Analytical Studio Express Server automates the processing of acquired data and, with the use of customized scoring algorithms, pre-selects ideal SFC and HPLC methods for purification. The visualization features of Analytical Studio Professional- Compound QC, particularly the reconstructed extracted composite ion chromatograms of the target product ion and the unknowns, enables the chromatographer to quickly interpret the data for their screened samples. In addition, this visual data interpretation tool is beneficial in cases where multiple COI peaks and co-eluting impurities affect the decision-making process, which often leads to time-consuming identification of appropriate purification conditions. Overall, the implementation of automated data processing and method selection redirects the focus of the separation scientists to purification-centric tasks, and minimizes the need for additional method development.

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