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Drug Discovery and Development

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Extractables and Leachables Analysis from Laboratory Tubing

Using a Quadrupole Time of Flight Mass Spectrometer and SWATH® Acquisition

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The analysis requirements of extractable and leachable compounds from products that come in contact with pharmaceutical products are described in the United States Pharmacopeia, chapters 1663 and 1664. The initial determination of non-volatile, organic extractable and leachable compounds is often done by high-resolution liquid chromatography-mass spectrometry using a time of flight (TOF) mass spectrometer run in Information Dependent Acquisition (IDA) mode. If compounds are present at low levels, or chromatographically coelute with other compounds of interest, they may be missed using IDA. Here, an LC-MS/MS method using SWATH acquisition is presented, that when coupled with an extractables and leachables (E&L) focused compound library, will provide greater confidence that compounds of interest are not missed during analysis.

Information dependent acquisition techniques have been available for users of tandem mass spectrometers for some time. These methods will typically select a precursor mass for fragmentation based on an algorithm that continuously evaluates intensity of all ions over a specified range. When an ion's intensity crosses a threshold, or otherwise satisfies the conditions set in the method, it will be isolated and fragmented to generate MS/MS spectra that can be used for identification or elucidation. Typically, the most intense ion at a given time will be isolated and fragmented, providing a spectra. If less intense, but still interesting, ions are present at the same retention time, those will often not be fragmented, and not therefore identified. SWATH acquisition is a data *independent* scan type that works by stepping through a series of Q1 m/z windows, fragmenting all of the ions transmitted through Q1, without a bias for intensity.[1] This approach allows coeluting compounds, even those present at very different levels, to have spectra acquired the first time a sample is run, and allow the analyst to have more confidence that important compounds are not missed.

Key Features of the X500R QTOF System for E&L Analysis

- Sensitive, fast scanning, benchtop hybrid quadrupole time of flight mass spectrometer
- SWATH acquisition enabled for the most comprehensive data independent analysis
  - Retention time alignment deconvolution when processing the data provides clean spectra that can be used for structure elucidation or library searching.
- Richly featured, user friendly, SCIEX OS software for acquisition and data processing for quantitative and qualitative workflows
- Extractable and Leachable focused NIST subset library of industry standard spectra and compounds that is user customizable
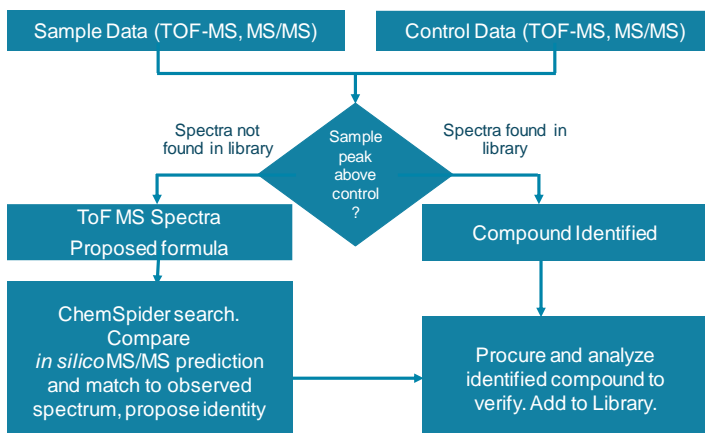
Figure 1. Compound Identification Workflow. Two strategies are used in this method for data processing to ensure the majority of compounds are detected and identified.

Methods

Sample Description: A sample of laboratory tubing soaked it acetone was generously provided by our collaborator.

Chromatography: Seperation was performed on an ExionLC™ AD HPLC system with a photodiode array detector (PDA 200-400nm) using a Phenomenex Luna Omega c18 column (1.6µm, 150x2.1mm). See Table 1 for more details.

Table 1. Chromatographic Conditions. Flow rate of 0.4 mL/min.

Time	% Mobile Phase A	% Mobile Phase B
0	98	2
1	98	2
12	0	100
16	0	100
16.2	98	2
18.2	98	2

Mobile phase A: Water with 0.01% formic acid and 3mM ammonium formate.
Mobile phase B: 90%CH_3CN 10% water with 0.01% formic acid and 3mM ammonium formate.

Mass Spectrometry: Data acquisition was performed using SCIEX X500R QTOF mass spectrometer operating in SWATH acquisition mode. A survey MS TOF scan from 100-1300 m/z, accumulation time 50ms is acquired followed by ten evenly divided SWATH acquisition windows (50 msec each) covering 100 – 1300 m/z. See Table 2 for SWATH acquisition windows. The mass range of each MS/MS was 50-1300 m/z with collision energy of 35 V and collision energy spread of 15 V. The resulting cycle time was ~0.6 seconds. Generic ESI source conditions were used in both polarities (GS1=40, GS2=40, CUR=25, ISV = +5500 / -5500).

Data Processing: All data was processed using the Sample vs Control qualitative workflow in SCIEX OS Analytics software. Using this workflow, sample and control injections are compared to each other after the peak finding algorithm is run, and only peaks in the sample that show areas greater than those found in the control by a threshold factor set in the method (i.e. 3x), are displayed for further evaluation. Two levels of processing were used in the method for this example. First, a targeted extraction list was included in the method. This list contained ~300 m/z values for known compounds. Second is using a non-targeted peak finding algorithm which identifies and integrates signals from the TOF MS data that are identified as peaks. Once peaks are identified by either method, the corresponding MS/MS

Table 2. SWATH Acquisition Windows.

Window	Q1 Start m/z	Q1 Stop m/z
1	100	220
2	219	340
3	339	460
4	459	580
5	579	700
6	699	820
7	819	940
8	939	1060
9	1059	1180
10	1179	1300

spectra are searched against a library for identification. If an acquired spectra is not matched to a library spectra, further evaluation of the peak can be done through the direct integration of SCIEX OS software with ChemSpider. A molecular formula is proposed based on the exact mass of the precursor, that formula is searched against ChemSpider which returns potential hits which include *.mol files. *In silico* fragmentation of the structures are compared to the acquired MS/MS, and the best match is selected by the user as the proposed compound. Figure 1 shows a schematic of the identification workflow.

The XIC list used for this work is based on a proprietary collection of historically identified compound from a collaborator. These lists are straightforward to generate and modify. They include a compound name and molecular formula, and ideally retention time if known. The extraction mass is calculated by the software, and the presence of chromatographic peaks is evaluated.

The library currently used is a curated subset of the 12,000 compound NIST MS/MS library. The list of included spectra is based on feedback from several sources globally to include only compounds relevant for this E&L application. The current version of this library contains ~3,000 spectra. New compounds and spectra can easily be added to a library by users, which allows library coverage to improve over time.
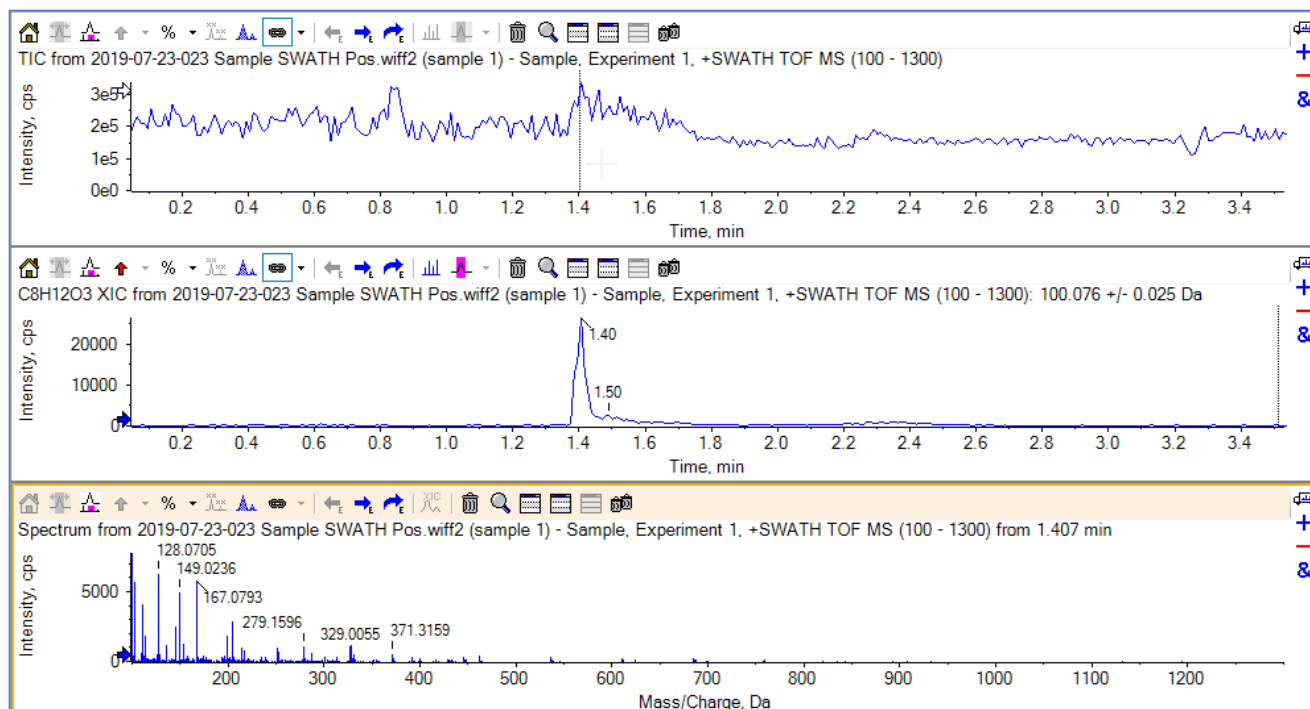
Figure 2. Advantage of SWATH Acquisition for Low Level Compound Detection. (Top) Total Ion Chromatogram (TIC) of TOF MS data where peak is barely visible. (Middle) Extracted Ion Chromatogram (XIC) of m/z 100.076, a compound of interest found by the processing method. (Bottom) TOF MS showing the complexity of the compounds eluting at RT 1.40. The low abundance of this ion at 100.076 m/z likely means it would have not generated MS/MS data in an IDA scan. Using SWATH acquisition, MS/MS was collected on all detectable precursors and therefore could be matched to the library for potential identification.

Detecting Low Abundant Compounds

The supplied acetone tubing extract sample, meant to be a part of a leachables study, was processed using the non-target screening method with a collaborator supplied XIC list, and the curated NIST library. Many polymer degradant peaks listed in the supplied XIC list were observed in the sample, as well as several compounds from the NIST library. There were also some peaks that were flagged for further investigation using the ChemSpider functionality. A few different examples of identified compounds will be shown to highlight the workflows.

SWATH acquisition analysis was chosen for this workflow to minimize the chances of missing a low abundance, but still interesting component. The identification of cyclohexylamine illustrates this. Figure 2 shows the total ion chromatogram of the TOF MS data, the extracted ion chromatogram for m/z 100.076 and the TOF MS data at 1.4 minutes. The peak finding algorithm was able to identify m/z 100.076 as a peak of interest compared with the control, but the TOF MS spectra at this retention time was quite complex. The compound's MS signal was very small relative to other signals and therefore would have been likely missed during IDA acquisition, not triggering collection of an MS/MS spectrum to use for potential identification. Using

SWATH acquisition, an MS/MS spectra is collected on all detectable compounds and therefore an MS/MS was available for library searching and was matched to the library entry for cyclohexylamine. See Figure 3 for a mirror plot of the library spectra match.
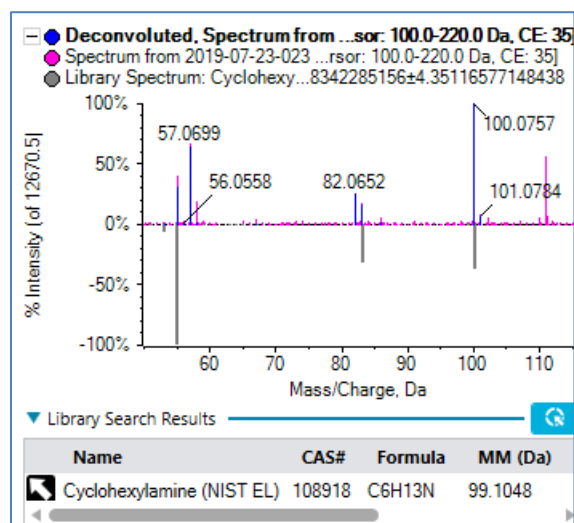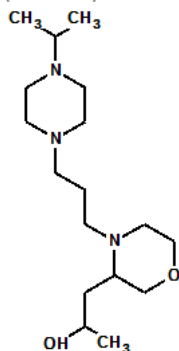

Figure 3. Identification of Cyclohexylamine. Mirror plot of the acquired spectra for m/z 100.076 compared with the library spectra for cyclohexylamine.
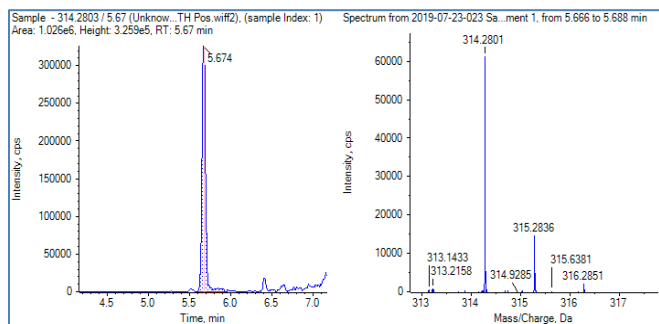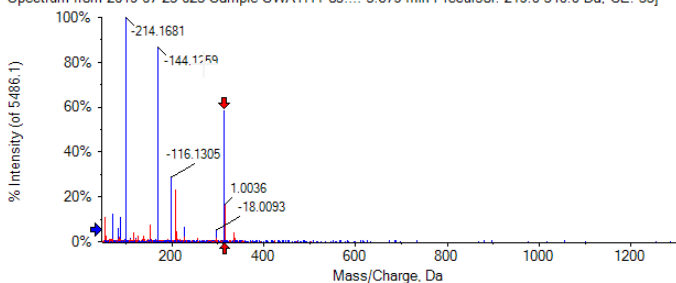
Figure 4. Unidentified Compound from Library Search. Component identified in the sample as interesting but providing no library match.

A peak identified at RT 5.67 (Figure 4) is an example of a peak found in the sample, whose spectra did not return a library match. The automatic formula finder calculates a formula of $C_{17}H_{35}N_2O_2$ with an error of less than 1ppm. When this formula is searched against ChemSpider, over 500 possible compounds


Figure 5. ChemSpider Results for m/z 314.28. Proposed structure, MS/MS and table listing *in-silico* fragments and their alignment to observed MS/MS fragments. Observed fragments not assigned are shown in orange.

are returned. The process of identifying an unknown peak without a library match is at best a challenging process. Even though there are many possible compounds associated with the chemical formula, the interface with ChemSpider and association of a structure with the collected MS/MS does help make the task less daunting. After quickly scrolling through the list of potential compounds, a couple of structures were found that corresponded well to the observed fragmentation (Figure 5).

Confirmation of this compound would involve investigating with the tubing sample vendor to see if it makes sense based on their manufacturing, storage or packaging processes, and potentially procuring an authentic standard for analysis and direct comparison with the sample results. An MS/MS spectra could then be added to the library for easier identification in the future.

Polymer Fragments and Degradants

One of the necessary and complex aspects to analyzing extractable compounds from pharmaceutical storage products is the confident identification of polymer fragments and degradants. Some classes of polymers require analysis by GC based techniques. For others, like nylon-based polymers that incorporate nitrogen into their structure, LC-MS/MS can be used. This can still be challenging because in many cases authentic standards are not commercially available, and many potential variations based on the polymer backbone can be observed. Further work is being done to develop a more comprehensive approach to identify and characterize this class of compounds, but currently, suspected degradants can be tentatively identified using chemical formulas that correspond to known or suspected combinations of the polymer monomers. Figure 6 below shows a probable nylon degradant peak found in the sample.
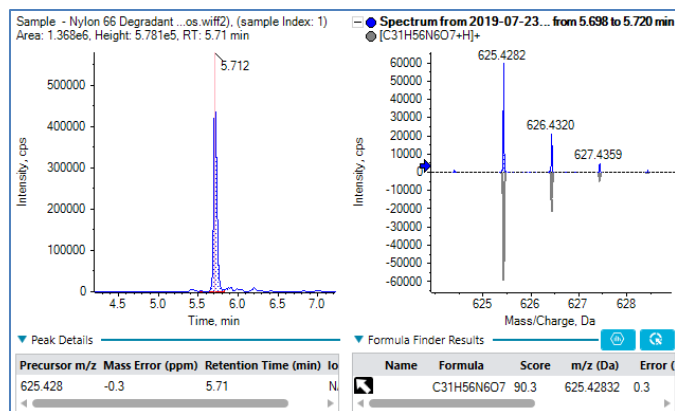

Figure 6. Nylon Degradant Found By Sample Control Comparison. XIC for a nylon degradant found in the sample (not present in the control) with its associated TOF MS shown as a mirror lot with the calculated MS of the formula (Right).

Optical Detection

Optical detection techniques (UV, PDA) are often incorporated into LC-MS based workflows to ensure that compounds that are not ionized during electrospray ionization are not missed as part of a study. In this case study, there were no significant peaks seen in the PDA trace that were not identified in the MS survey for the sample. This is not surprising being that modern LC-MS/MS is generally much more sensitive than most optical techniques. Figure 7 below shows the PDA and MS signals for a bis-2-ethylhexyl phthalate standard, which did show a good response by both techniques. Untargeted peak finding using the optical signal can be done in SCIEX OS software.

Conclusions

Here, a workflow and example data for a high-resolution LC-MS/MS extractables and leachables method is demonstrated that can be implemented using the SCIEX X500R QTOF system. The method incorporates a photodiode array detector to allow non-ionizable compounds to also be detected. A curated version of the NIST Tandem Mass Spectral library is used for identification of high-resolution MS/MS spectra. Users can easily add compound spectra to their library, which allows compound coverage for their assay to grow in a focused manner.
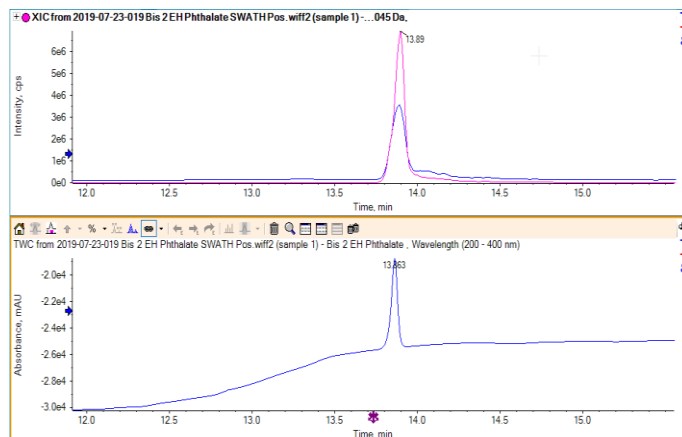
Figure 7. Example of Optical Detection. (Top) TOF MS XIC and MS/MS XIC from SWATH acquisition experiment for bis-2-ethylhexyl phthalate standard. (Bottom) Total Wavelength Chromatogram, showing >2 second RT offset.

References

1. Improved Data Quality Using Variable Q1 Window Widths in SWATH® Acquisition - Data Independent Acquisition on TripleTOF® and X-Series QTOF Systems. SCIEX Technical note RUO-MKT-02-2879-B.