Investigating the metabolomic changes induced from long term drug abuse

Using SWATH acquisition on the X500R QTOF system and Polly by Elucidata

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The combination of chromatography with tandem mass spectrometry (for example, LC-MS/MS, LC-QTOF-MS) has been an indispensable tool in the forensic toxicology laboratory to investigate the presence of drugs in a variety of complex biological matrices. LC-MS typically results in the identification and quantification of these substances with high levels of sensitivity and specificity. In recent years, the adoption of high-resolution mass spectrometry (HRMS) has opened new avenues for the development of screening and conformation workflows that acquire comprehensive MS/MS spectral fragment information on every detectable component in the sample using data-independent analysis.

In addition to providing valuable information about the type of drugs and drug metabolites present in a sample, the use of SWATH acquisition provides a snapshot of the individual's metabolome, which reflects their biochemical activity at the time of sample collection. Studying the endogenous metabolites, exogenous compounds, conjugates, hormones, etc. provides a wealth of information that can uncover meaningful insights into the effects of drug consumption on human biology.

One of the bottlenecks when processing large-scale metabolomic datasets collected with SWATH acquisition is the computing power required to deeply interrogate the data. The transition from raw data to meaningful insights requires many steps, from peak detection, curation and identification to further downstream analysis, to derive meaningful biological insights.

In this study, the Polly platform by Elucidata was used as a comprehensive end-to-end processing platform for the analysis of a cohort of 75 forensic urine samples collected using SWATH acquisition. The results of this study showcase the platform's ability to seamlessly curate metabolomic features extracted from a large dataset, providing valuable insights into the metabolomic features shared amongst the various groups of drug users represented in the sample cohort.

Key advantages of an end-to-end metabolomics data processing platform

- SWATH acquisition is a powerful data acquisition method that generates comprehensive and high-quality MS/MS spectra of all detectable analytes, providing a digital record of a sample in a single injection
- The Polly platform provides a single and integrated platform for data processing, storage and management
- The Polly platform is compatible with the analysis of large SWATH acquisition datasets and allows data processing

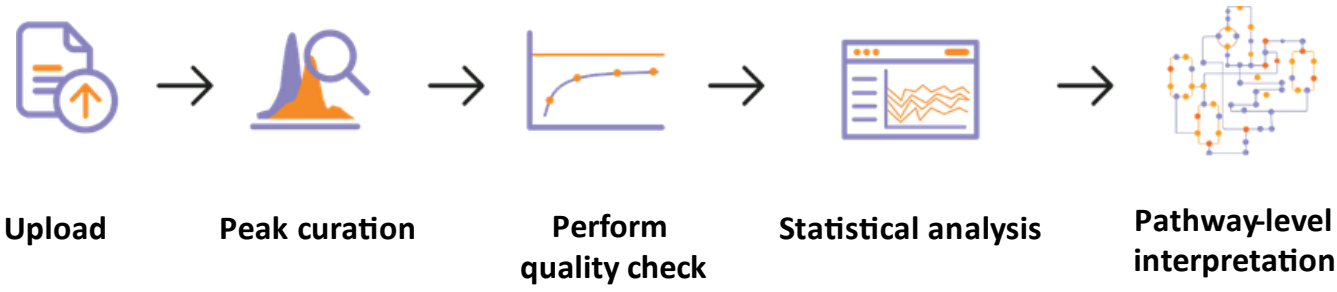

Figure 1. Data processing workflow for metabolomics SWATH acquisition data using Polly. Typical workflow using the Polly platform includes data upload to the Polly EI-MAVEN, peak curation and quality checks, followed by statistical analysis (differential expression) and pathway-level interpretation.

customization, alleviating the need for multiple software tools

- A typical workflow includes data upload, peak curation, quality check and statistical analysis leading to pathway-level interpretation (Figure 1)
- Large sample sets were analyzed using Polly El-MAVEN and Dual-Mode Data Visualization (Metabolomics) App on Polly
- Downstream analysis of the data included normalization, quality checks, statistical analysis and comparative analysis with intuitive and interactive visualizations, such as pathway-level interpretation of the data
- Interpretation of the data highlighted the differences in metabolite expression for each cohort, providing valuable insights on the correlation between metabolic changes and the resulting pathway alteration

Experimental details

Samples description: A cohort of 75 forensic urine samples, including 21 control samples and 54 samples that tested positive for at least 1 psychoactive substance, were analyzed in this study. The 54 positive samples were further divided into 3 sub-groups: positive to fentanyl, positive to opioids and positive to other drugs. The 4 sample types (control and positive samples) were labeled as follows: NegativeControl, PositiveToFentanyl, PositiveToOpioids and PositiveToOtherDrugs.

Sample preparation: Urine samples were centrifuged at 13.3 g for 5 minutes and the supernatant was diluted 1:4 with the gradient's starting mobile phase mixture to reach a final volume of 100 µL. Quality control (QC) samples consisted of blank urine spiked with the internal standard fentanyl-D5 at a final concentration of 500 ng/mL after dilution with the mobile phase.

Chromatography: HPLC separation was achieved using a Phenomenex C18 column (100 x 2.1 mm, 1.7 µm, 00D-4475-AN) held at 45°C on the ExionLC AC system. Mobile phases used consisted of water and acetonitrile, each with modifiers. The LC flow rate was 0.5 mL/min and the total run time was 11.5 min. The injection volume was 5 µL.

Mass spectrometry: MS and MS/MS data were collected using SWATH acquisition on the X500R QTOF system with SCIEX OS software 1.5. MS and MS/MS data were acquired using positive electrospray ionization mode. Data acquisition consisted of a TOF MS scan followed by 12 MS/MS scans using variably sized Q1 windows. Mass ranges for the TOF MS and TOF MS/MS experiments were 100-1000 m/z and 50-1000 m/z, respectively.

Data analysis: Data processing was performed using Elucidata's Polly™ platform. An end-to-end customized pipeline was employed for the processing and analysis of the SWATH acquisition data. Polly El-MAVEN was selected as the data processing application for the large (50 GB) dataset comprised of 75 forensic toxicology urine samples. The workflow consisted of data upload, peak picking and quality check, followed by statistical analysis (for example, differential expression) and pathway-level interpretation of the results on the Polly Dual-Mode Data Visualization (Metabolomics) Application. The workflow used for the analysis of the samples is summarized in Figure 1.

Peak curation and quality checks

Data were collected using SWATH acquisition on the cohort of 75 forensic urine samples. These raw, unprocessed data were uploaded to the Polly El-MAVEN application for data processing. A targeted feature detection (for example, peak picking and integration) was performed using an exhaustive spectral library with more than 11,000 spectra. From this analysis, 152 features were detected, 110 of which were unique. Further analysis was then performed using the Polly Dual-Mode Data Visualization (Metabolomics) App. The raw peak areas of the detected features were normalized for downstream statistical analysis. Figure 2 shows the density plots and boxplots A) before and B) after data normalization. The density plots and boxplots show a normal distribution of the data across the samples following data normalization.

Following normalization, multivariate statistical analysis was performed on the dataset as part of the quality checks. Principal component analysis (PCA) was used to identify trends, cluster samples and find outliers across the 4 different sample groups.

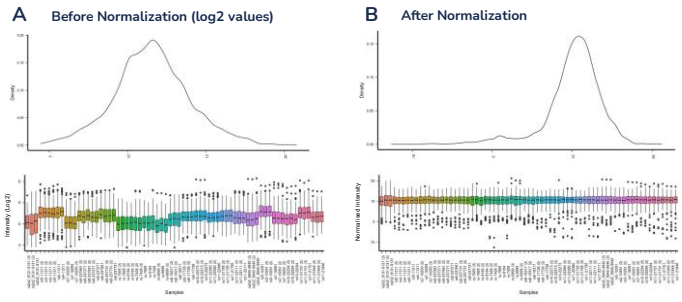

Figure 2. Visualization of data normalization in Elucidata's Polly platform. Density plots (top) and boxplots (bottom) represent the data distribution A) before and B) after normalization. Data normalization reduces errors across a dataset and ensures data are stored logically for subsequent statistical analysis.

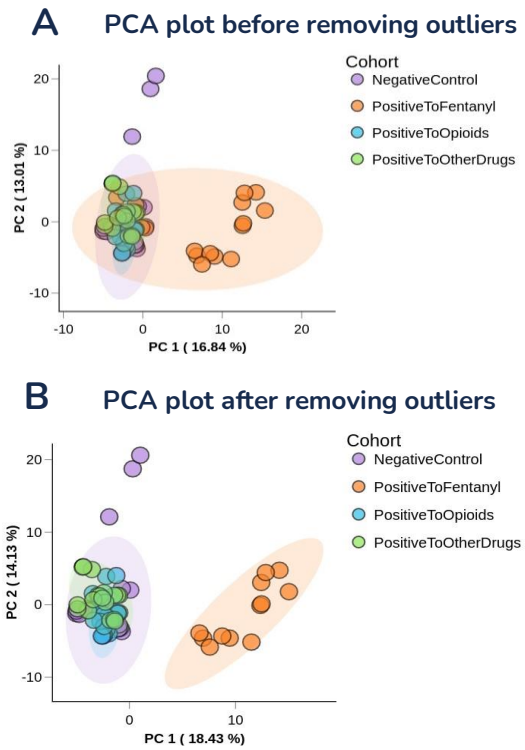

Figure 3. PCA scores plots for the 4 different sample groups. PCA was performed on the normalized dataset A) before and B) after removing 2 outlier samples belonging to the PositiveToFentanyl group. The scores plots show that the PositivetoFentanyl sample category separated out well from the other 3 sample groups.

Figure 3 shows the PCA scores plots of the second versus the first principal components for the normalized data across the 4 different sample groups A) before and B) after outlier removal. A comparison between 3A and 3B reveals that removal of the outliers resulted in tighter clustering of the groups. As a result, the identified outlier samples belonging to the PositiveToFentanyl sample group were removed from downstream analysis. These PCA scores plots suggest that the PositivetoFentanyl samples can be distinguished from the other 3 sample groups.

Following removal of the outlier samples, PCA plots were generated to compare the PositivetoFentanyl sample category to the other 3 sample types. Figure 4 shows the PCA scores plots of the first and second principal components for the PositivetoFentanyl sample category plotted against each of the 3 other sample types. The PCA scores plots show that the PositivetoFentanyl samples clearly cluster together with good reproducibility and the cluster separates significantly when compared to the other groups. This suggests the presence of unique spectral features that enable differentiation of the samples belonging to this PostivetoFentanyl groups from the other 3 sample types.

Statistical analysis

Differential expression analysis was performed to compare the abundance level of detected metabolites between the PositiveToFentanyl samples and the other samples. Statistics were applied to assess the significance of any differences detected. These analyses were implemented to identify unique metabolomic signatures that could differentiate the sample groups and correlate with the type of drug consumed.

Figure 5A shows a volcano plot comparing the PositiveToFentanyl sample group to the PositiveToOtherDrugs group. The plot shows the statistical significance (p value on the y-axis) as a function of the magnitude of the differences in expression values of the samples in the groups (fold changes, expressed as log2FC on the x-axis. The plot enables visual identification of the features that are different between groups and are statistically relevant. A p value cutoff of 0.05 and log2FC threshold of 0.5 were used to identify the 102 significant features marked in red.

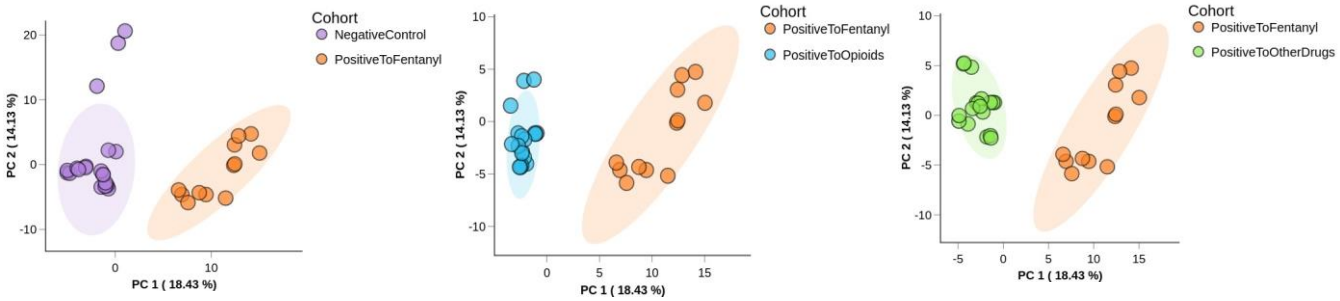

Figure 4. PCA scores plots comparing PositiveToFentanyl samples to the other 3 sample types. PCA was performed to compare PositiveToFentanyl against A) NegativeControl, B) PositiveToOpioids and C) PositiveToOtherDrugs. The PCA scores plots demonstrate separation of PositiveToFentanyl from the other 3 sample types. This observation provides statistical evidence for the presence of unique spectral features differentiating this sample type from the others.

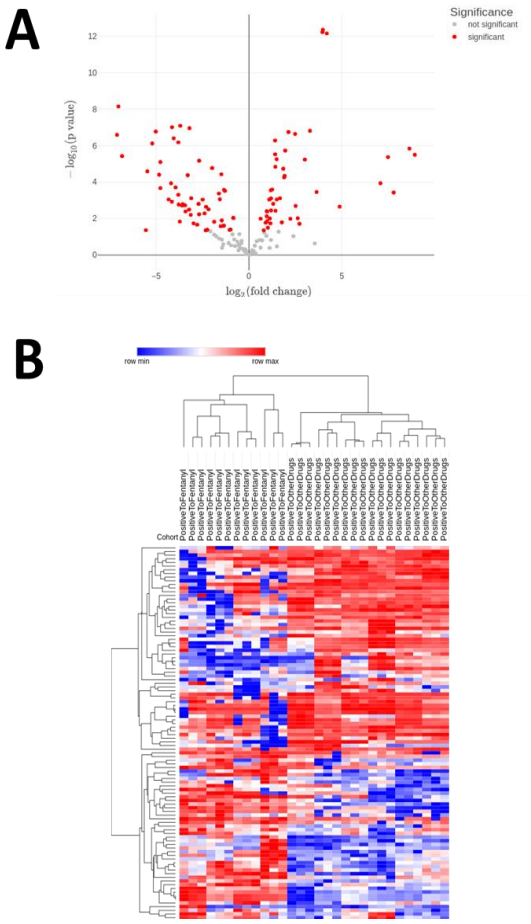

Figure 5. Statistical results from the comparison of the PositiveToFentanyl group to the PositiveToOtherDrugs group. A) Volcano plot highlighting the 102 significant features in red from the PositiveToFentanyl vs. PositiveToOtherDrugs comparison. B) Heatmap with hierarchical clustering analysis for the identified significant features.

Figure 5B shows a hierarchically clustered heatmap of log2FC values of the 102 significant features across the samples belonging to the 2 sample types. The red and blue represent increased and decreased abundance in PositiveToFentanyl samples, respectively. The color density denotes the level of fold change.

Pathway analysis

Pathway analysis is a powerful tool that helps place experimental observations into a relevant biological context. Interpreting the relative abundances of metabolites across samples at the pathway level is often crucial to draw biological conclusions. Enrichment analysis provides insight into the pathways that are most perturbed due to the biological changes under investigation. Figure 6A shows the enriched metabolic sets that are significantly different between the PositiveToFentanyl samples and the PositiveToOtherDrugs samples. The pathway topology plot shown in Figure 6B shows that the pathways involved in 1) phenylalanine, tyrosine and tryptophan biosynthesis, 2) phenylalanine metabolism and 3) histidine metabolism are the most perturbed pathways in the comparison between the PositiveToFentanyl and PositiveToOtherDrugs samples. The plots displayed in Figure 6 provide insights into the effect of fentanyl consumption compared to other drug consumption on human metabolism and offer specific information about the metabolic differences between fentanyl users and other drug users.

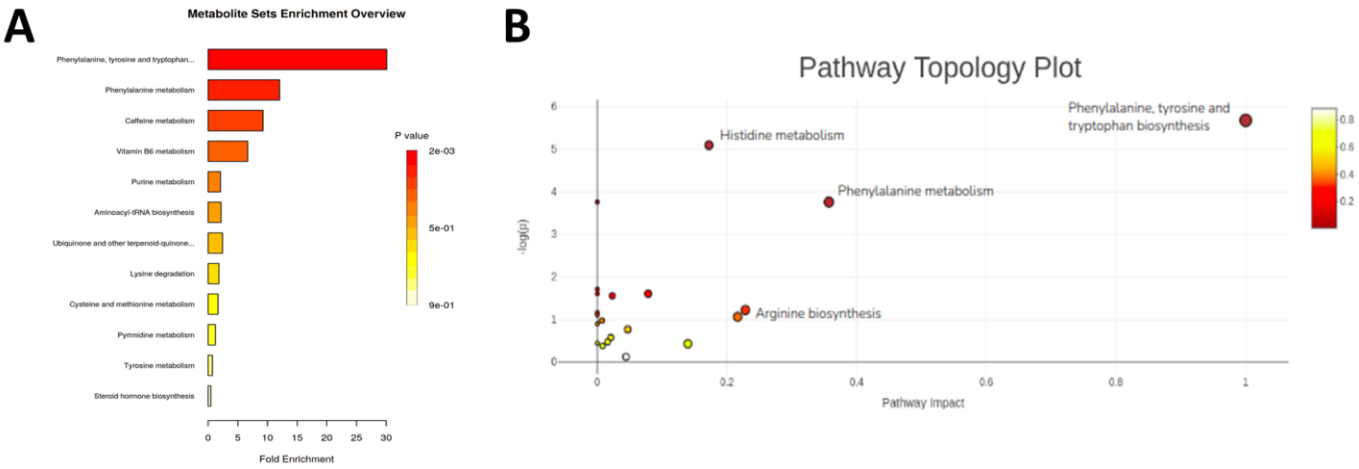

Figure 6. Graphical representations of pathway analysis for the comparison between the PositiveToFentanyl and the PositiveToOtherDrugs sample types. A) Enrichment plot provides an overview of the metabolite sets responsible for the differentiation between the PositiveToFentanyl and PositiveToOtherDrugs groups. B) Pathway topology plot shows the most enriched pathways in the comparison between the PositiveToFentanyl and the PositiveToOtherDrugs groups. The groups of metabolites highlighted in these 2 plots provide insights into the biological pathways that are most perturbed by the consumption of fentanyl vs. other drugs.

Comparison between multiple sample types

The statistical tools available in the Elucidata's Polly platform enabled interactive visualization of the dataset, showcasing metabolic differences between the sample groups. To further complement the comparison of different samples, a cross-sample comparison was performed using the information extracted from the pathway analysis. This comparison provides a deeper understanding of the metabolites that differ or are shared between the sample groups included in this dataset. Figure 7 shows an UpSet plot that helps visualize the common/overlapping features found to be different relative to PositiveToFentanyl in the other 3 samples. The single dots in the plot highlight the metabolites that are unique to specific comparisons, for example, sample group vs. PositiveToFentanyl. The lines highlight the metabolites that were found to be different in multiple other samples relative to the PositiveToFentanyl samples.

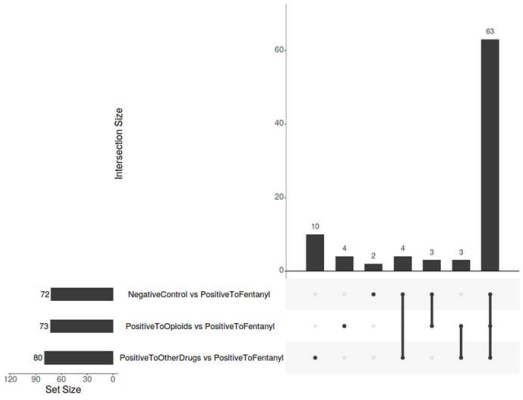

Figure 7. UpSet plot showing the unique and common features across the compared samples. The plot provides a graphical representation of the intersecting features amongst the compared sample groups.

Table 1 summarizes the results from the UpSet plot and lists the number and name of the unique metabolites shared between the PositiveToFentanyl sample group and each of the 3 other sample groups. The metabolites provide unique indicators of the common biological impact shared amongst the different drug users' groups. Harnessing this information provides meaningful biological insight into the effect of drug consumption on the overall biology and metabolome of the various types of drug users.

Table 1. List of the unique metabolites shared between the PositiveToFentanyl samples and each of the three other sample groups represented in the cohort.

Sample type comparison	Number of metabolites	Name of metabolites
unique to PositiveToFentanyl vs. PositiveToOtherDrugs	10	4-Formyl-antipyrine, 5'-Methylthioadenosine, L-Norvaline, Codeine, Phenylephrine, Caffeine, Propamocarb, Amitriptyline , Dehydroepiandrosterone, Rebemide
unique to PositiveToFentanyl vs. PositiveToOpioids	4	D-Isoleucine, Primidone, (S)-Carnitine, Oseltamivir-carboxylate
unique to PositiveToFentanyl vs. NegativeControl	2	trans-2-Hydroxycinnamate, Cymoxanil

Conclusions

In this study, a large cohort made of 75 urine samples collected from subjects who previously tested positive to different types of drugs was analyzed using SWATH acquisition on a X500R QTOF system. With this analysis workflow, both MS and MS/MS data were collected on all detectable species, enabling confident detection and quantification of both exogenous drugs as well as endogenous metabolites in a single analysis. Next, Elucidata's Polly platform was used to analyze the dataset, identifying detected metabolites, quantifying their differences across the samples and providing statistical analysis to find unique metabolic signatures between the different groups of drug users represented in the sample cohort.

- The Polly platform provided a comprehensive data processing platform for targeted metabolomics using SWATH acquisition
- Interpretation of the data in the Polly El-MAVEN and Dual-Mode Data Visualization (Metabolomics) application highlighted the differences in metabolite abundance observed between the sample types, providing valuable insights on the resulting pathway alterations
- A complete metabolomics workflow from data collection to results analysis using SWATH acquisition and Polly platform proved capable of gaining biological insight into the effects of drug consumption on the metabolome and overall human biology

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