Nontargeted acquisition with targeted and suspect screening of pharmaceutical drugs and their metabolites in wastewater

Simultaneous quantitation and screening by the SCIEX X500R QTOF system and SCIEX OS software

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Introduction

This technical note describes the identification of pharmaceutical drugs and their metabolites in wastewater using nontargeted acquisition coupled with suspect screening. A solid-phase extraction (SPE) LC-MS/MS method was developed for the semi-quantitative screening of 105 pharmaceutical drugs. The X500R QTOF system was used to collect MS/MS data by SWATH data-independent acquisition (DIA). These data were used to identify targeted drug compounds and retrospectively detect previously untargeted metabolites from a combined approach of spectral library matching and diagnostic fragment confirmation. Molecule Profiler software provided a complementary workflow for metabolite identification by matching common fragments against those from *in silico* fragmentation.

Wastewater monitoring has been increasingly adopted to assess community drug exposure due to its low costs, non-invasive sample collection and comprehensive analytical coverage.[1] In contrast, drug epidemiological data derived from self-reported surveys and toxicological reports can be expensive and be biased from the lack of or skewed responses from the sampled populations. SWATH DIA produces high-resolution MS/MS spectra that are composites of all analytes present in the sample and can be retrospectively mined.

Here, an end-to-end workflow using the X500R QTOF system and integrated modules within the SCIEX OS software provided high-resolution MS and MS/MS data for targeted and non-targeted screening of drugs and their metabolites in wastewater environments. Figure 1 shows the identification of carbamazepine and its metabolites based on complementary approaches of MS/MS library matching and *in silico* fragment confirmation in the Molecule Profiler software module of SCIEX OS software.

Key features of SWATH DIA on the X500R QTOF system coupled with targeted and nontargeted screening with SCIEX OS software

- SWATH DIA acquisition on the X500R QTOF system provided comprehensive MS/MS coverage for both targeted and nontargeted screening of all compounds
- Integration of the Analytics module and Molecule Profiler software within SCIEX OS software enabled a seamless transition between spectral library matching and *in silico* fragmentation predictions for compound identification in a single software platform
- A SPE LC-MS/MS workflow enabled the simultaneous semi-quantitation and identification of 105 pharmaceutical drugs in small volumes of wastewater samples

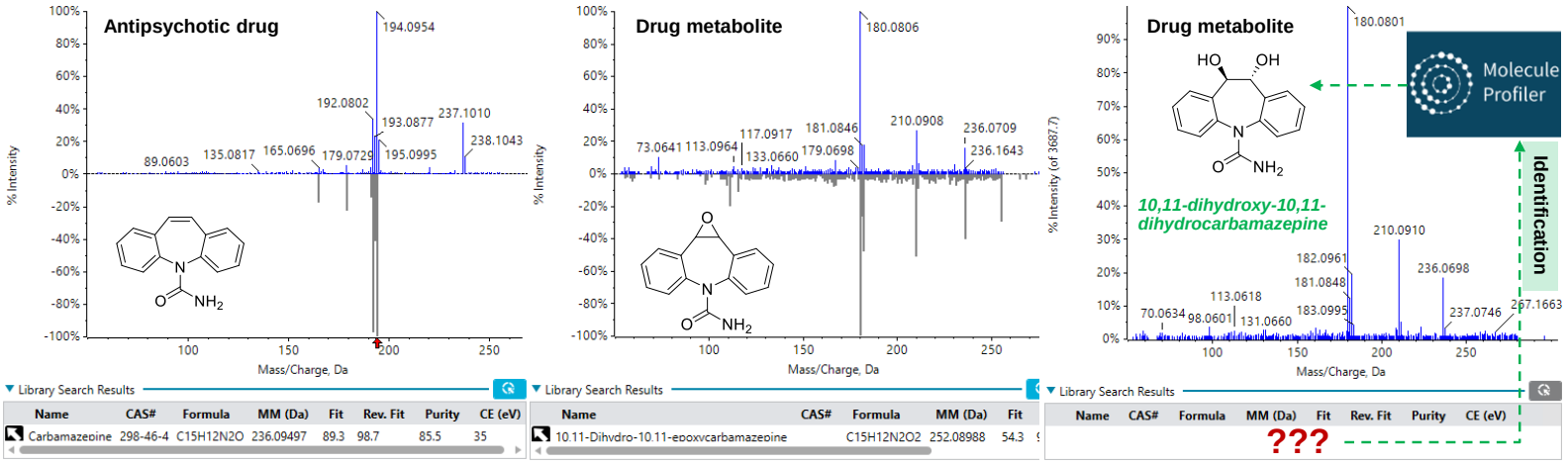


Figure 1. Identification of different targeted pharmaceutical drugs and drug metabolites that were not initially targeted for acquisition. Retrospective analysis of SWATH DIA MS/MS data revealed the detection of several metabolites of carbamazepine via a combined approach of spectral library matching and *in silico* structural elucidation in the integrated Molecule Profiler software and Analytics module of SCIEX OS software.

Experimental methods

Chemicals and samples: The target analyte list included 105 pharmaceutical drugs and 3 surrogate internal standards. Individual neat standards were mixed to prepare stock solutions in methanol from which calibration standards (5–1000 ng/L) were prepared in MilliQ water for semi-quantitation. Influent wastewater samples were collected as 24-hour composites from 4 sites in the northwestern region of Italy. Upon collection, a 1 L aliquot of composite wastewater was transferred to refrigerated glass bottles and stored at -20°C until analysis.

Sample preparation: A 100 mL sample of wastewater was centrifuged at 4000 rpm for 5 minutes and vacuum-filtered through a 0.22 µm filter. A 30 mL aliquot of filtered wastewater was spiked with the surrogate internal standards and extracted using an Oasis HLB SPE cartridge (200 mg, 6 cm^3, Waters, Milford, MA). Each cartridge was preconditioned with 5 mL of methanol and 5 mL of MilliQ water before loading the sample, then was vacuum dried and eluted with 10 mL of methanol. Upon evaporation to dryness, the residue was reconstituted with 50 µL of methanol for LC-MS/MS analysis. Spiked MilliQ water was prepared in the same manner for semi-quantitative assessment of limits of detection (LOD) and extraction recoveries.

Chromatography: LC separation was performed on a SCIEX ExionLC AC system using a Phenomenex Kinetex C18 column (100 x 2.1 mm, 1.7 µm, P/N: 00D-4475-AN). A flow rate of 0.5 mL/min, an injection volume of 5 µL and a column temperature of 45°C were used. The LC conditions used are shown in Table 1.

Mass spectrometry: Analysis was performed using the X500R QTOF system in both positive and negative electrospray ionization mode. Table 2 shows the method parameters used for the mass spectrometer. The SWATH DIA method consisted of 16 variable windows covering a mass range of *m/z* 130–520.

Table 1. Chromatographic gradient.

Time (min)	%A	%B
0.0	*95*	*5*
0.5	*95*	*5*
8.0	*5*	*95*
8.5	*5*	*95*
8.51	*95*	*5*
11	*95*	*5*

Mobile phase A: MilliQ water with 5mM formic acid
Mobile phase B: Acetonitrile with 5mM formic acid

Table 2. Source, gas and temperature conditions.

Parameter	MS	MS/MS
Polarity	*Positive and negative*	
Ion spray voltage	*2500 V*	
Ion source gas 1 (GS1)	*50 psi*	
Ion source gas 2 (GS2)	*45 psi*	
Curtain gas (CUR)	*35 psi*	
Collision gas (CAD)	*8 psi*	
Source temperature (TEM)	*600°C*	
Declustering potential (DP)	*65 V*	
Total scan time	*0.836 s*	
Scan mode	*TOF MS*	*SWATH DIA*
Start/stop mass range	*130 – 520 Da*	*50 – 800 Da*
Accumulation time	*0.25 s*	*0.03 s*
Collision energy (CE)	*10 V*	*35 V*
Collision energy spread (CES)	*0 V*	*15 V*

Data processing: Data were acquired and processed using SCIEX OS software, versions 2.2 and 3.1. A custom library of previously acquired MS/MS spectra was used for library searching. The Molecule Profiler software was used to screen for drug metabolites. Figure 2 shows the overall workflow.

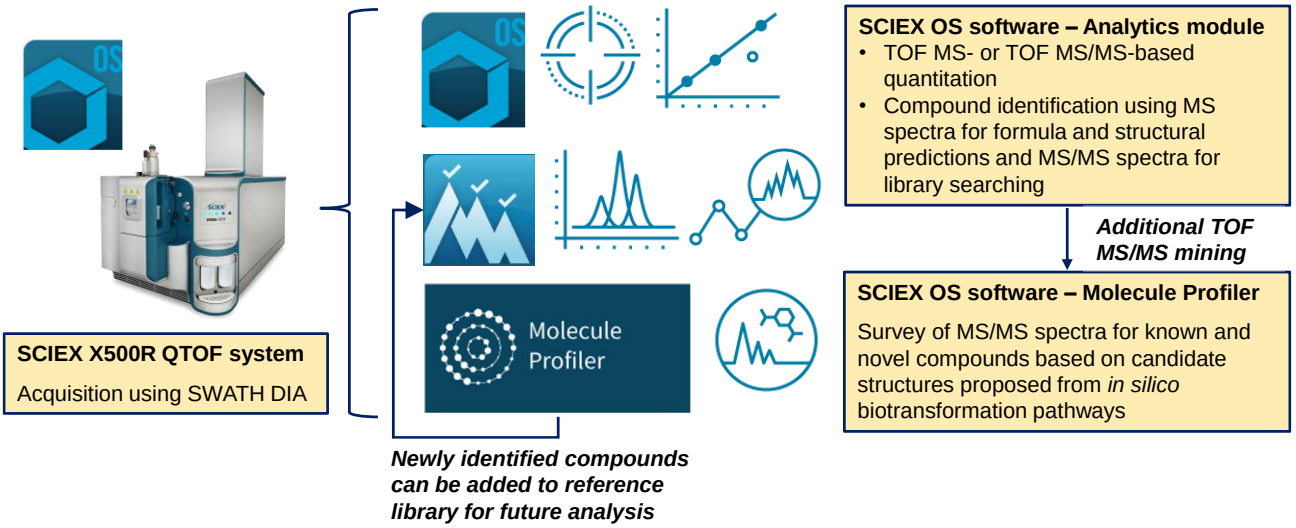


Figure 2. Streamlined acquisition and data analysis workflow using the X500R QTOF system and SCIEX OS software. The Analytics module was used for quantitation and spectral library matching, while Molecule Profiler software was used for metabolite identification.

Targeted analysis of drugs in wastewater

In contrast to the 100–250 mL samples typically used in SPE methods for wastewater analysis, only 30 mL was extracted here, which reduced solvent consumption and the matrix load. The SPE LC-MS/MS method achieved recoveries of ≥70% for 60% of the 105 targeted drugs and 50–70% for most of the remaining analytes based on comparisons of pre- and post-extracted aqueous spikes. Based on aqueous spikes exhibiting signal-to-noise (S/N) ratios ≥3 above the background, the instrumental LODs were estimated to be ≤5 ng/L for 72% of the analytes and 5–15 ng/L for the remainder, consistent with the typical concentration ranges of drugs observed in wastewater. As such, the developed SPE LC-MS/MS method provided acceptable performance based on fit-for-purpose criteria for the semi-quantitation of a large panel of targeted analytes in wastewater influents (Table 3). Table 3 shows the average concentration range for a subset of the 105 targeted drugs detected in wastewater whereby compound identification in each sample was confirmed by retention time (RT) matches against authentic standards, mass error of <5 ppm for the exact precursor and fragment *m/z* peaks, and spectral MS/MS matching against a custom library of previously acquired MS/MS spectra using

Table 3. Compound information for a subset of the 105 targeted pharmaceutical drugs detected in wastewater influents. Chemical formula, adduct, assigned internal standard, precursor and fragment ion *m/z*, retention times (RT), limits of detection (LOD), extraction recovery (RE) and the range of average concentrations reported from the 4 wastewater treatment plants (WWTPs) are included.

Compound	Formula	Adduct	Internal standard	Precursor *m/z*	Fragment *m/z*	RT (min)	LOD (ng/L)	RE (%)	Avg conc range (ng/L) from 4 sites
Antidepressants									
Citalopram	$C_{20}H_{21}FN_2O$	$[M+H]^+$	*Cocaine-d3*	*325.1711*	*109.0453*	*3.84*	*5*	*101*	*nd – 220*
Mirtazapine	$C_{17}H_{19}N_3$	$[M+H]^+$	*Nitrazepam-d5*	*266.1652*	*195.0915*	*2.90*	*5*	*63*	*nd – 23*
Trazodone	$C_{19}H_{22}ClN_5O$	$[M+H]^+$	*Nitrazepam-d5*	*372.1586*	*176.0804*	*3.30*	*5*	*74*	*5 – 19*
Benzodiazepine									
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	$[M+H]^+$	*Nitrazepam-d5*	*321.0192*	*275.0144*	*4.20*	*5*	*91*	*24 – 160*
Lormetazepam	$C_{16}H_{12}Cl_2N_2O_2$	$[M+H]^+$	*Nitrazepam-d5*	*335.0349*	*289.0286*	*4.62*	*5*	*82*	*9 – 160*
Oxazepam	$C_{15}H_{11}ClN_2O_2$	$[M+H]^+$	*Nitrazepam-d5*	*287.0582*	*241.0528*	*4.09*	*5*	*96*	*nd – 36*
Temazepam	$C_{16}H_{13}ClN_2O_2$	$[M+H]^+$	*Nitrazepam-d5*	*301.0738*	*255.0679*	*4.47*	*5*	*90*	*nd – 8*
Antipsychotic									
Amisulpride	$C_{17}H_{27}N_3O_4S$	$[M+H]^+$	*Cocaine-d3*	*370.1795*	*242.0477*	*2.55*	*5*	*67*	*nd – 120*
Carbamazepine	$C_{15}H_{12}N_2O$	$[M+H]^+$	*Nitrazepam-d5*	*237.1022*	*194.0949*	*3.90*	*5*	*82*	*100 – 600*
Quetiapine	$C_{21}H_{25}N_3O_2S$	$[M+H]^+$	*Nitrazepam-d5*	*384.1740*	*253.0795*	*3.63*	*5*	*70*	*nd – 39*
Tiapride	$C_{15}H_{24}N_2O_4S$	$[M+H]^+$	*Cocaine-d3*	*329.1530*	*256.0615*	*1.98*	*5*	*97*	*nd – 5*
Venlafaxine	$C_{17}H_{27}NO_2$	$[M+H]^+$	*Cocaine-d3*	*278.2115*	*58.0656*	*3.25*	*5*	*67*	*nd – >1000*
Antiepileptic									
Lamotrigine	$C_9H_7Cl_2N_5$	$[M+H]^+$	*Nitrazepam-d5*	*256.0151*	*210.9820*	*2.73*	*15*	*69*	*nd – 860*
Oxcarbazepine	$C_{15}H_{12}N_2O_2$	$[M+H]^+$	*Nitrazepam-d5*	*253.0972*	*180.0810*	*3.58*	*5*	*91*	*nd – 380*
Cardiovascular drugs									
Atenolol	$C_{14}H_{22}N_2O_3$	$[M+H]^+$	*Cocaine-d3*	*267.1703*	*145.0638*	*1.60*	*5*	*46*	*nd – 500*
Bisoprolol	$C_{18}H_{31}NO_4$	$[M+H]^+$	*Nitrazepam-d5*	*326.2326*	*116.1068*	*3.38*	*5*	*57*	*25 – 77*
Nebivolol	$C_{22}H_{25}F_2NO_4$	$[M+H]^+$	*Nitrazepam-d5*	*406.1824*	*151.0561*	*4.24*	*5*	*56*	*nd – 68*
Propafenone	$C_{21}H_{27}NO_3$	$[M+H]^+$	*Nitrazepam-d5*	*342.2064*	*116.1067*	*4.12*	*5*	*77*	*30 – 220*
Ramipril	$C_{23}H_{32}N_2O_5$	$[M+H]^+$	*Cocaine-d3*	*417.2384*	*234.1497*	*3.97*	*5*	*67*	*nd – 26*
Telmisartan	$C_{33}H_{30}N_4O_2$	$[M+H]^+$	*Nitrazepam-d5*	*515.2442*	*497.2324*	*4.54*	*5*	*86*	*nd – 350*
Non-steroidal anti-inflammatory drugs									
Ketoprofen	$C_{16}H_{14}O_3$	$[M+H]^+$	*Coumachlor*	*255.1016*	*105.0328*	*4.58*	*5*	*76*	*48 – 900*
Analgesic/opioids									
Paracetamol	$C_8H_9NO_2$	$[M+H]^+$	*Coumachlor*	*152.0706*	*110.0604*	*1.53*	*10*	*80*	*nd – >1000*
Tapentadol	$C_{14}H_{23}NO$	$[M+H]^+$	*Cocaine-d3*	*222.1852*	*107.0488*	*2.90*	*5*	*90*	*44 – 380*
Others									
Dextromethorphan	$C_{18}H_{25}NO$	$[M+H]^+$	*Cocaine-d3*	*272.2009*	*215.1416*	*3.59*	*5*	*83*	*nd – 260*
Gliclazide	$C_{15}H_{21}N_3O_3S$	$[M+H]^+$	*Cocaine-d3*	*324.1376*	*127.1225*	*4.86*	*5*	*83*	*nd – 180*
Lidocaine	$C_{14}H_{22}N_2O$	$[M+H]^+$	*Cocaine-d3*	*235.1805*	*86.0965*	*2.49*	*5*	*77*	*43 – >1000*
Metoclopramide	$C_{14}H_{22}ClN_3O_2$	$[M+H]^+$	*Cocaine-d3*	*300.1473*	*227.0586*	*2.72*	*5*	*75*	*nd – 19*

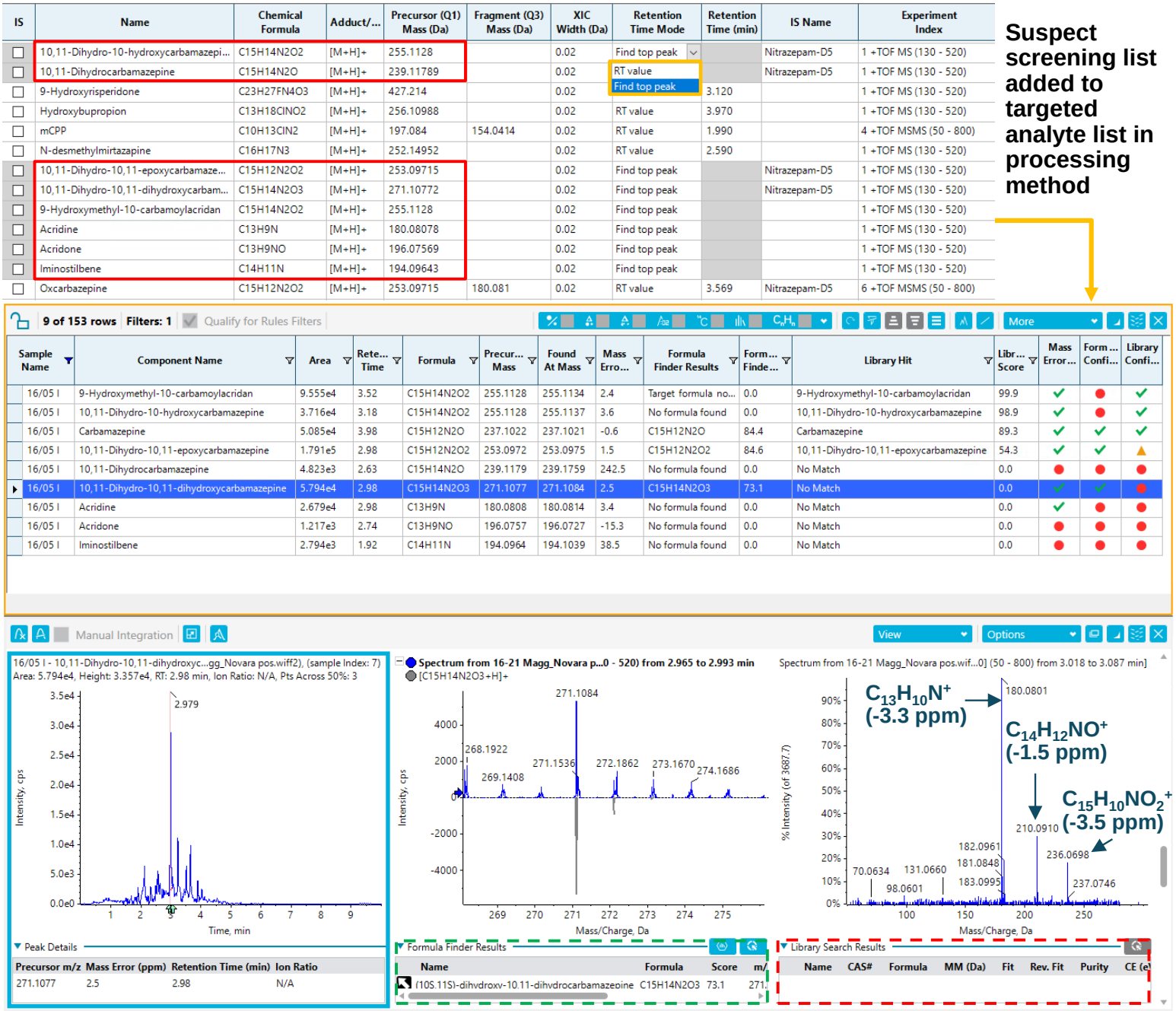
nd = not detected

reference standards. The traffic light system in the SCIEX OS software expedites data review by enabling the user to filter and display only the results passing predefined confidence thresholds for identification, such as mass error and matches in RT, isotope ratio pattern and MS/MS spectra against a library, as shown by some example positive hits in Figure 3.

In addition to confirming positive detection of the parent drugs, monitoring their metabolites has become increasingly prevalent, since specific metabolites have demonstrated toxicity comparable to their parent drugs.

Suspect screening for previously untargeted metabolites using SCIEX OS software

Non-targeted acquisition by SWATH DIA enabled retrospective analysis of TOF MS/MS to screen for previously untargeted compounds, such as the metabolites of positive drug hits in wastewater. Due to its well-documented metabolic pathways,[2-4] carbamazepine (CBZ) was used as the model parent drug to screen for metabolites that were not initially targeted. The molecular formula and exact precursor masses of 8 known CBZ metabolites were determined *a priori* from the literature to



IS	Name	Chemical Formula	Adduct/...	Precursor (Q1) Mass (Da)	Fragment (Q3) Mass (Da)	XIC Width (Da)	Retention Time Mode	Retention Time (min)	IS Name	Experiment Index
☐	10,11-Dihydro-10-hydroxycarbamazepi...	C15H14N2O2	[M+H]+	255.1128		0.02	Find top peak		Nitrazepam-D5	1 +TOF MS (130 - 520)
☐	10,11-Dihydrocarbamazepine	C15H14N2O	[M+H]+	239.11789		0.02	RT value / Find top peak		Nitrazepam-D5	1 +TOF MS (130 - 520)
☐	9-Hydroxyrisperidone	C23H27FN4O3	[M+H]+	427.214		0.02		3.120		1 +TOF MS (130 - 520)
☐	Hydroxybupropion	C13H18ClNO2	[M+H]+	256.10988		0.02	RT value	3.970		1 +TOF MS (130 - 520)
☐	mCPP	C10H13ClN2	[M+H]+	197.084	154.0414	0.02	RT value	1.990		4 +TOF MSMS (50 - 800)
☐	N-desmethylmirtazapine	C16H17N3	[M+H]+	252.14952		0.02	RT value	2.590		1 +TOF MS (130 - 520)
☐	10,11-Dihydro-10,11-epoxycarbamaze...	C15H12N2O2	[M+H]+	253.09715		0.02	Find top peak		Nitrazepam-D5	1 +TOF MS (130 - 520)
☐	10,11-Dihydro-10,11-dihydroxycarbam...	C15H14N2O3	[M+H]+	271.10772		0.02	Find top peak		Nitrazepam-D5	1 +TOF MS (130 - 520)
☐	9-Hydroxymethyl-10-carbamoylacridan	C15H14N2O2	[M+H]+	255.1128		0.02	Find top peak			1 +TOF MS (130 - 520)
☐	Acridine	C13H9N	[M+H]+	180.08078		0.02	Find top peak			1 +TOF MS (130 - 520)
☐	Acridone	C13H9NO	[M+H]+	196.07569		0.02	Find top peak			1 +TOF MS (130 - 520)
☐	Iminostilbene	C14H11N	[M+H]+	194.09643		0.02	Find top peak			1 +TOF MS (130 - 520)
☐	Oxcarbazepine	C15H12N2O2	[M+H]+	253.09715	180.081	0.02	RT value	3.569	Nitrazepam-D5	6 +TOF MSMS (50 - 800)

Sample Name	Component Name	Area	Rete... Time	Formula	Precur... Mass	Found At Mass	Mass Erro...	Formula Finder Results	Form... Finde...	Library Hit	Libr... Score	Mass Error...	Form... Confi...	Library Confi...
16/05 I	9-Hydroxymethyl-10-carbamoylacridan	9.555e4	3.52	C15H14N2O2	255.1128	255.1134	2.4	Target formula no...	0.0	9-Hydroxymethyl-10-carbamoylacridan	99.9	✓	●	✓
16/05 I	10,11-Dihydro-10-hydroxycarbamazepine	3.716e4	3.18	C15H14N2O2	255.1128	255.1137	3.6	No formula found	0.0	10,11-Dihydro-10-hydroxycarbamazepine	98.9	✓	●	✓
16/05 I	Carbamazepine	5.085e4	3.98	C15H12N2O	237.1022	237.1021	-0.6	C15H12N2O	84.4	Carbamazepine	89.3	✓	✓	✓
16/05 I	10,11-Dihydro-10,11-epoxycarbamazepine	1.791e5	2.98	C15H12N2O2	253.0972	253.0975	1.5	C15H12N2O2	84.6	10,11-Dihydro-10,11-epoxycarbamazepine	54.3	✓	✓	▲
16/05 I	10,11-Dihydrocarbamazepine	4.823e3	2.63	C15H14N2O	239.1179	239.1759	242.5	No formula found	0.0	No Match	0.0	●	●	●
16/05 I	10,11-Dihydro-10,11-dihydroxycarbamazepine	5.794e4	2.98	C15H14N2O3	271.1077	271.1084	2.5	C15H14N2O3	73.1	No Match	0.0	✓	✓	●
16/05 I	Acridine	2.679e4	2.98	C13H9N	180.0808	180.0814	3.4	No formula found	0.0	No Match	0.0	✓	●	●
16/05 I	Acridone	1.217e3	2.74	C13H9NO	196.0757	196.0727	-15.3	No formula found	0.0	No Match	0.0	●	●	●
16/05 I	Iminostilbene	2.794e3	1.92	C14H11N	194.0964	194.1039	38.5	No formula found	0.0	No Match	0.0	●	●	●

Figure 3. Suspect screening for the metabolites of carbamazepine (CBZ) in a wastewater sample. The top panel shows the targeted components list in the processing method with the suspect CBZ metabolites added. The bottom panel shows the results table filtered to display CBZ and its suspect metabolites. Formula Finder identified 10,11-dihydro-10,11-dihydroxycarbamazepine (DiOH-CBZ) (highlighted in green), but further confirmation by library searching was not possible due to the absence of this compound in the reference library (highlighted in red). Instead, DiOH-CBZ was identified based on diagnostic fragment comparisons against published MS/MS spectra.

p 4

generate a suspect screening list in the processing method (Figure 3). The RT mode was selected for these suspect compounds with unknown RTs to "Find top peak" to identify the most intense peak eluting at a specific RT within the extracted ion chromatogram (XIC). Three metabolites, 10,11- dihydro-10-hydroxycarbamazepine (10-OH-CBZ), 10,11-dihydro-10,11-dihydroxycarbamazepine (DiOH-CBZ) and carbamazepine 10,11-epoxide (EP-CBZ) were identified based on mass error (<5 ppm), isotope ratio and spectral matching against a custom library (Figure 3). Although DiOH-CBZ was not present in the custom library, its predominant fragments of $[C_{13}H_{10}N]^+$, $[C_{14}H_{12}NO]^+$ and $[C_{15}H_{10}NO_2]^+$ were present with good mass error (<5 ppm), which is consistent with MS/MS spectra reported in published databases.[5] In addition, Formula Finder predicted several candidate formulas based on the MS and MS/MS spectra, one of which matched the structure of DiOH-CBZ found in the ChemSpider database (Figure 4) .

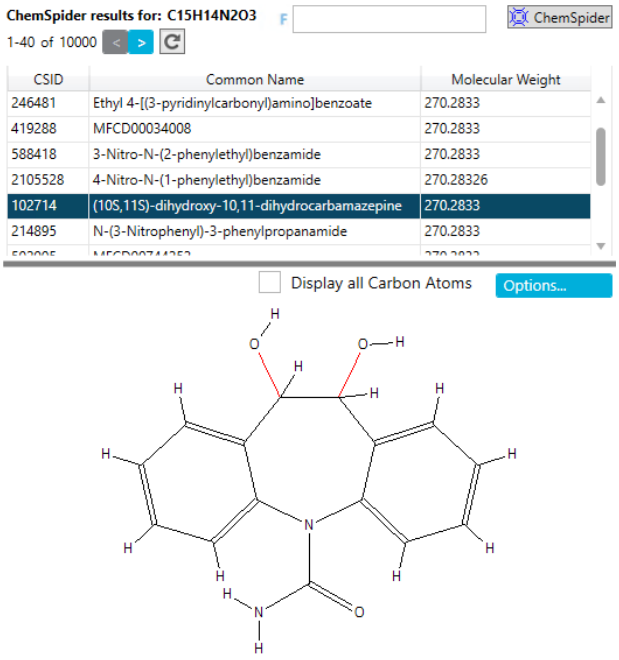


Figure 4. Identification of DiOH-CBZ using Formula Finder and ChemSpider in the Analytics module of SCIEX OS software. Based on the experimental MS and MS/MS spectra, Formula Finder generated a list of candidate formulas and searched them against structures in the ChemSpider database. The experimental MS/MS spectrum matched the *in silico* predicted fragmentation of the candidate structure of DiOH-CBZ.

A limitation of this workflow is that it required *a priori* knowledge of the molecular formula and/or exact precursor mass *m/z* of the compounds to be targeted for suspect screening. This demands an exhaustive search in the literature to produce a comprehensive list of suspect metabolites, which can be time-consuming and labor-intensive. As such, some of the wastewater samples were reinterrogated using the Molecule Profiler module to corroborate these findings here and screen for additional metabolites that may have been missed from suspect screening.

Detection of additional CBZ metabolites using Molecule Profiler software

The Molecule Profiler software in SCIEX OS software provided an orthogonal workflow for detecting metabolites by searching for precursor compounds that also share characteristic fragments from the parent CBZ structure such as *m/z* 194.0941, 192.0795 and 179.0725. These fragments are commonly observed in the MS/MS spectra of CBZ metabolites in the published literature.[1-4] As shown in Figure 5, the software used *in silico* biotransformation pathways to predict a list of expected cleavage metabolites, such as DiOH-CBZ, which could not be previously confirmed by MS/MS library matching due to its absence in the reference spectral library. Table 4 shows a list of metabolites identified based on good mass error (<5 ppm) and comparison between *in silico* fragmentation of the predicted candidate structure and the MS/MS spectra. In addition to the same metabolites found by the Analytics module, Molecule Profiler software tentatively identified additional metabolites such as $C_{14}H_{13}NO_3$ and $C_{15}H_{12}N_2O_2$ that were not previously targeted.

For example, a monohydroxycarbamazepine structure was predicted for the candidate compound $C_{15}H_{12}N_2O_2$, observed at *m/z* 253.0979 at a RT of 3.31 minutes. This peak was separate from its other structural isomers, EP-CBZ and oxcarbazepine (OX-CBZ), which elute at 2.98 and 3.98 minutes, respectively. All 3 isomers lose the carboxamide group ($CONH_3$) to produce the fragment pairs at *m/z* 210.091 and 208.076. EP-CBZ and OX-CBZ have been reported to produce additional major fragments at *m/z* 236.071 and 180.081, which were not observed in the experimental MS/MS spectrum here.[2,4] The lack of a reference MS/MS spectrum for library confirmation precluded further confirmation of the exact positional isomer of the monohydroxycarbamazepine.

Overall, the Molecule Profiler software identified similar metabolites found by suspect screening in the Analytics module of SCIEX OS software and tentatively identified others, all without *a priori* knowledge of the analyte details. Both modules provide complementary approaches such as MS/MS library matching and *in silico*-based fragmentation pattern prediction to aid in the discovery of known and novel metabolites. The integration of Molecule Profiler software with SCIEX OS software enables the user to seamlessly transport their metabolite findings to the Analytics module for further library confirmation and updates of their spectral library with any novel metabolites identified, as shown in Figure 2.

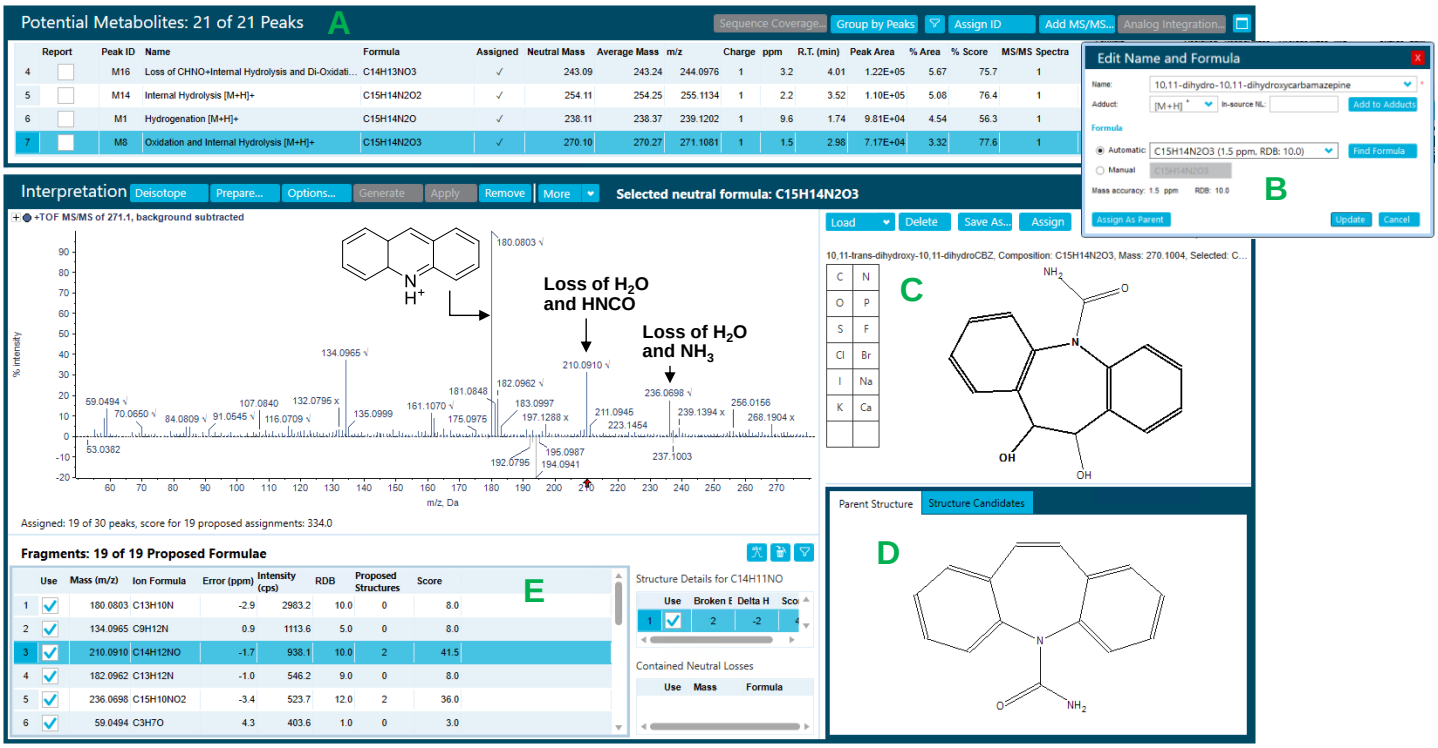


Figure 5. Identification of DiOH-CBZ, a CBZ metabolite, using Molecule Profiler software. The software displays a list of potential metabolites with their formula, *m/z* and scoring (A) with the ability to edit compound details (B). The Interpretation panel enables the user to review and compare candidate structures for the metabolite (C) and parent (D). The software also allows users to edit and assign new structures based on annotated fragment peaks in the TOF MS/MS spectrum (E).

Conclusions

- SWATH DIA of MS/MS spectra enables retrospective mining of previously acquired data for drug metabolites that were not targeted during the initial pharmaceutical drug screen
- Accuracies of ≥70% and LODs of ≤5 ng/L for the majority of the targeted drugs were achieved based on solvent spikes and were deemed adequate for the semi-quantitative screening of 105 pharmaceutical drugs in wastewater using SPE LC-MS/MS
- Molecule Profiler software provided an automated workflow for metabolite identification without *a priori* knowledge of the analyte details for processing
- Integration of Molecule Profiler software with SCIEX OS software enabled a streamlined workflow for transferring metabolite findings to be orthogonally confirmed by interrogation of the MS/MS spectra using diagnostic fragment ions and library searching in the Analytics module of SCIEX OS software

References

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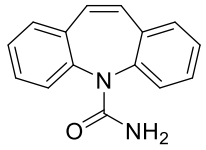
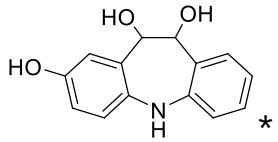
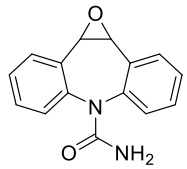
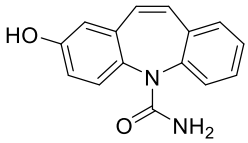
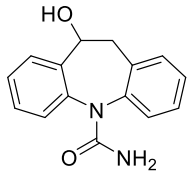
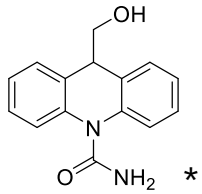
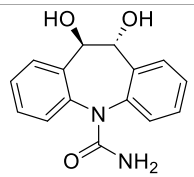
Table 4. List of parent CBZ and proposed metabolites identified in a wastewater sample using Molecule Profiler software. Each proposed metabolite predicted by a biotransformation pathway is highlighted in orange based on identification from the molecular formula of the precursor and fragment ions, the precursor and fragment mass error (<5 ppm), the software-assigned structure, RT and % score that indicates the likelihood that the peak found is a metabolite.

Biotransformation pathway (Compound)	Molecular formula	Structure	Precursor *m/z* (Error, ppm)	Fragment *m/z* (Error, ppm)	Fragment formula	RT (min)	% Score	Found in Analytics module via suspect screening
Parent $[M+H]^+$ *(Carbamazepine)*	$C_{15}H_{12}N_2O$		*237.1020 (-0.9)*	*194.0956 (-4.2)* *179.0730 (0.3)*	$[C_{14}H_{12}N]^+$ $[C_{13}H_9N]^+$	*3.99*	*82.5*	*Yes*
Loss of CHNO+ internal hydrolysis and di-oxidation $[M+H]^+$ *(Unknown)*	$C_{14}H_{13}NO_3$	 *	*244.0976 (3.2)*	*194.0967 (1.7)* *192.0807 (-0.2)*	$[C_{14}H_{12}N]^+$ $[C_{14}H_{10}N]^+$	*4.01*	*75.7*	*No*
Oxidation $[M+H]^+$ *(Carbamazepine-10,11-epoxide)*	$C_{15}H_{12}N_2O_2$		*253.0975 (1.4)*	*180.0806 (-0.9)* *210.0909 (-2.2)*	$[C_{13}H_{10}N]^+$ $[C_{14}H_{12}NO]^+$	*2.98*	*76.8*	*Yes*
Oxidation $[M+H]^+$ *(1-hydroxycarbamazepine, 2-hydroxycarbamazepine, 3-hydroxycarbamazepine)*	$C_{15}H_{12}N_2O_2$		*253.0979 (2.9)*	*210.0916 (1.2)* *208.0756 (-0.2)*	$[C_{14}H_{12}NO]^+$ $[C_{14}H_{10}NO]^+$	*3.31*	*74.3*	*No*
Internal hydrolysis $[M+H]^+$ *(10,11-dihydro-10-hydroxycarbamazepine)*	$C_{15}H_{14}N_2O_2$		*255.1136 (3.1)*	*194.0960 (-2.4)* *192.0809 (0.8)* *237.1022 (-0.2)*	$[C_{14}H_{12}N]^+$ $[C_{14}H_{10}N]^+$ $[C_{15}H_{13}N_2O]^+$	*3.17*	*74.7*	*Yes*
Internal hydrolysis $[M+H]^+$ *(9-hydroxymethyl-10-carbamoyl acridan)*	$C_{15}H_{14}N_2O_2$	 *	*255.1134 (2.2)*	*194.0962 (-1.2)* *180.0805 (-1.3)* *238.0869 (2.6)*	$[C_{14}H_{12}N]^+$ $[C_{13}H_{10}N]^+$ $[C_{15}H_{12}NO_2]^+$	*3.52*	*76.4*	*Yes*
Oxidation and internal hydrolysis $[M+H]^+$ *(10,11-dihydro-10,11-dihydroxycarbamazepine)*	$C_{15}H_{14}N_2O_3$		*271.1081 (1.5)*	*180.0803 (-2.9)* *210.0910 (-1.7)* *236.0698 (-3.4)*	$[C_{13}H_{10}N]^+$ $[C_{14}H_{12}NO]^+$ $[C_{15}H_{10}NO_2]^+$	*2.98*	*77.6*	*Yes*

*Structure was not predicted by Molecule Profiler software. The structure was instead drawn based on manual comparison between the experimental MS/MS and published MS/MS from the literature or inferred from the proposed biotransformation pathway used to predict that metabolite.

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