Verdel Instruments

Application Note Number WBE001

Identification of population and oxidative stress biomarkers in wastewater

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Key words: wastewater based epidemiology, public health, pharmaceuticals, oxidative stress

This is an application note and does not contain a detailed experimental section.

Abstract

WBE is an established technique for analysing public health but has largely been constrained to targeted analysis due to the complexity of wastewater. TOC-MS™ is a new data independent analytical technique that is ideally suited for analysis of complex mixtures. The TOC-MS method was able to identify twenty-one biomarkers in a single sample by combining targeted detection using standards, with untargeted identification through the use of retrospective data analysis.

Introduction

Wastewater based epidemiology (WBE) is a technique that seeks to understand public health through analysis of wastewater as a single surrogate, community-wide pooled urine sample (1) and has been widely used to monitor illicit drugs (2), compounds of environmental concern, such as pesticides (3), and pharmaceuticals (4) and health focused biomarkers, like biomarkers of oxidative stress (5; 6). The complexity of wastewater is what allows it to be used in so many potential applications. However, this complexity often necessitates the use of targeted or data dependent analysis (DDA) in order to identify and quantify molecules with low abundance. Where a data independent analysis (DIA) technique is used, it becomes more important that the LC-MS approaches, which are typically used for WBE, can effectively separate and isolate all analytes in order to avoid acquiring information about multiple analytes at once.

Total Correlation Mass Spectrometry (TOC-MS™) is a new MS analytical technique that aims to provide MS/MS data for every precursor in a spectra without requiring separation or isolation of analytes to effectively eliminate the production of chimeric spectra. TOC-MS is based on the existing principles of two dimensional mass spectrometry (7) (2D-MS), which controls how molecules fragment such that the fragments can be intrinsically linked to their original molecules without first separating them. As such TOC-MS is a data independent analysis (DIA) technique.

In brief, TOC-MS works by using direct infusion and electrospray ionisation to generate ions that can then be stored inside a linear ion trap. The position of these ions are then modulated relative to the centre of the trap, using radiofrequency pulses known as stored waveform ion radius modulation (SWIM). After modulation a UV laser is fired and passes through the centre of the linear ion trap. Molecules closer to the centre of the trap are more exposed to the laser and undergo ultraviolet photodissociation (UVPD) to produce fragments.

Over a series of SWIM pulses, as a precursor is moved in and out of the centre of the trap its intensity, and the intensity of any fragments produced by UVPD, will vary as a function of its m/z. In this way precursors and fragments can be intrinsically correlated, effectively eliminating the issue of chimeric spectra.

For WBE, the ability to get full MS/MS data for every precursor in a sample will allow for wastewater to be thoroughly profiled for the first time and allows for retrospective data analysis digitally. This will allow analysts to more easily identify novel biomarkers in wastewater alongside existing analytes in a single analytical experiment.

Methodology

Analytical standards and solvents of the highest possible purity were purchased from Sigma Aldrich. Biomarkers of population size were selected from a previous paper (8), which identified a positive correlation between biomarker concentration and population size, and included: acetaminophen, atenolol, caffeine, carbamazepine, codeine, furosemide, hydrochlorothiazide, ibuprofen, naproxen and salicylic acid. These included several pharmaceuticals and lifestyle chemicals that are routinely detected in wastewater. Biomarkers of oxidative stress were collated from several prior papers (6; 5) seeking to explore the correlation between oxidative stress and public health. The biomarkers selected were as follows: 5β-tetrahydrocortisol (5B-HYC), 8-Hydroxy-2'-deoxyguanosine (8-OHdG), 8-hydroxyguanosine, 8-nitroguanine, hydrocortisone, prostaglandin E2 (PE2) and prostaglandin F2α (PF2a). Biomarkers of oxidative stress were used as they can potentially be used to monitor overall public health through WBE. Two mixtures of each set of biomarkers were prepared in 100% methanol with a final concentration of 100 nM for each biomarker. One of each pair of mixtures was spiked with 0.1% v/v formic acid and the others were spiked with 0.1% ammonium hydroxide.

Influent wastewater was collected by grab sampling after coarse screening from the inlet of a sewage treatment works serving a large city in the South-West of the UK in 2021 and shipped to a nearby lab on ice for extraction. 100 mL of wastewater was filtered through Whatman GF/F filter paper and the filtrate loaded onto conditioned Oasis HLB SPE cartridges (3cc, 150 mg sorbent). Cartridges were conditioned using 2 mL of methanol followed by 2mL of milli-Q water. After loading, cartridges were washed with 3 mL of milli-Q water and left to dry under vacuum for 20 minutes. After drying cartridges were bundled and stored in foil at -20°C before being shipped to Verdel for analysis. Once arrived, cartridges were stored at -20°C until ready for analysis. Cartridges were eluted using 3 mL of methanol and each portion of eluent was divided into three 1 mL aliquots. The first was spiked with 0.1% v/v formic acid, the second with 0.1% v/v ammonium hydroxide and the third was stored at -20°C for future analysis. One set of aliquots were then spiked with either population size or oxidative stress biomarkers to a final concentration of 100 nM for each biomarkers.

Samples were infused directly into the TOC-MS instrument at a rate of 0.25 µL min^{-1} using a borosilicate glass, 22 gauge, 500 µL Hamilton syringe and a NE-1000 programmable single syringe pump. Samples containing formic acid were analysed in ESI+ mode, whilst samples containing ammonium hydroxide were analysed in ESI- mode.

MS source conditions: End plate offset voltage: 500 V, Capillary voltage: 4.0 kV, Nebulising gas pressure: 0.3 Bar, Dry gas flow rate: 4.0 mL min^{-1}, Dry gas temp: 200°C

UVPD parameters: Laser wavelength: 213 nm

Analytical data was displayed and interpreted using Verdel's *Data Analysis* software.

Results and discussion

For the population size biomarkers the initial analysis was performed using the mixtures in methanol in order to identify optimal ionisation conditions and UVPD fragmentation patterns. The TOC-MS spectra are shown in figures 1 and 2, and the results summarised in table 1. TOC-MS spectra are plotted as a top down view showing fragment m/z (from the ToF) on the X-axis and the precursor m/z (from the TOC-MS process) on the Y-axis. Fragments and their precursor will have the same coordinates in the Y-axis. The intensity of the detected ion is proportional to the darkness of the spot. In general, all biomarkers were detected in at least one ionisation mode and most had at least one fragment identified and structurally confirmed.

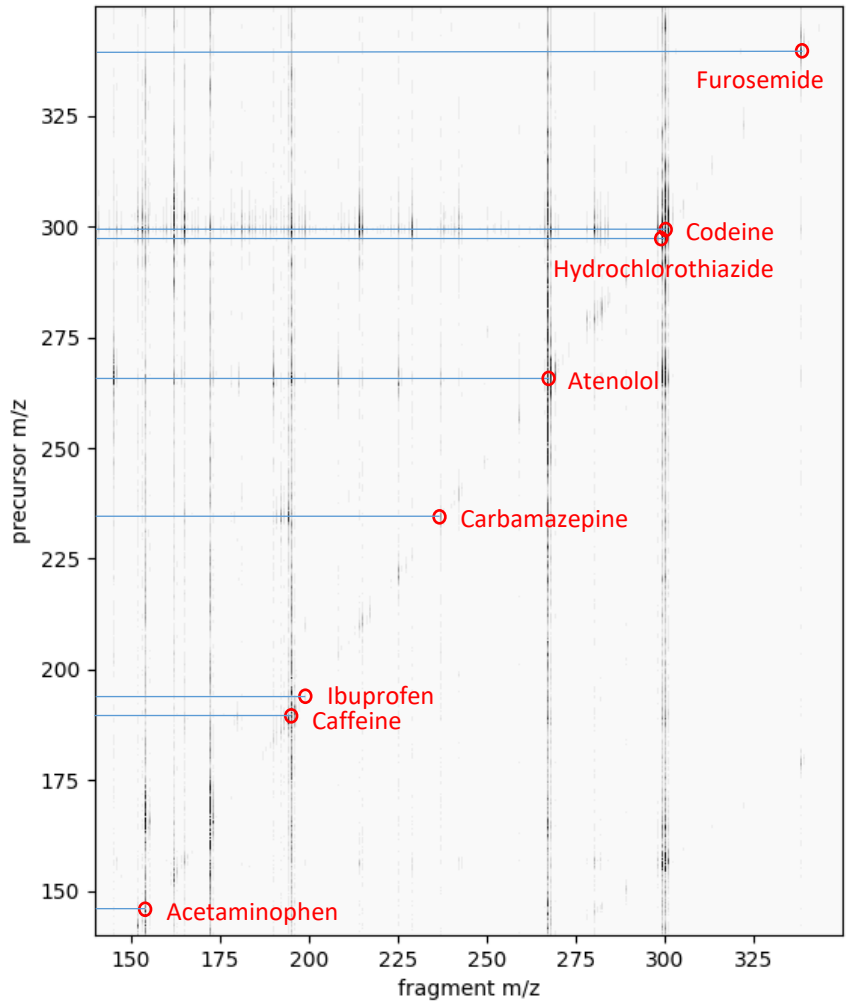

Figure 1. Biomarkers spiked into methanol – ESI+ analysis

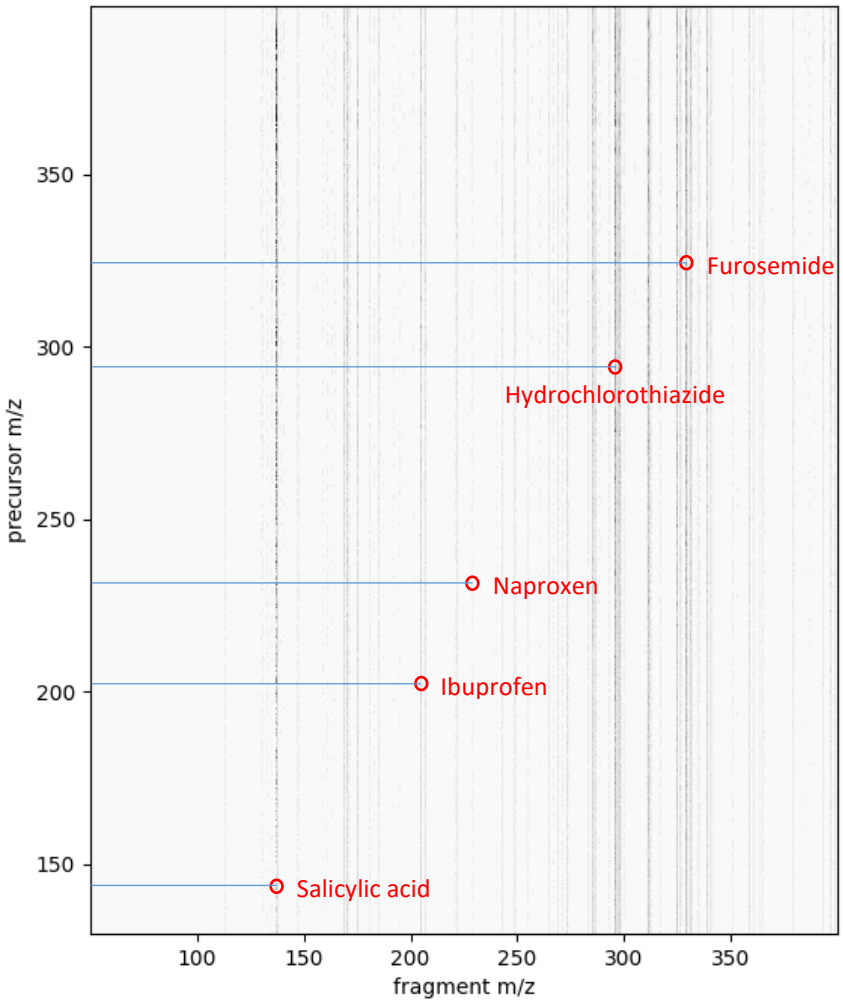

Figure 2. Biomarkers spiked into methanol – ESI- analysis

Table 1. Detected precursors and fragments of population equivalence biomarkers from analysis in methanol

Compound	Precursor mass	Ionisation mode	Fragment 1: m/z (formula)	Fragment 2: m/z (formula)	Fragment 3: m/z (formula)	Fragment 4: m/z (formula)
Salicylic acid	137.0248	Negative	121.039 ($C_7H_5O_2$)	93.029 (C_6H_5O)		
Acetaminophen	152.0699	Positive	138.059 ($C_7H_6NO_2$)	134.061 (C_8H_8NO)	93.030 (C_6H_5O)	68.026 (C_4H_4O)
Caffeine	195.0874	Positive	180.060 ($C_7H_8N_4O_2$)	138.067 ($C_6H_8N_3O$)	97.043 ($C_4H_5N_2O$)	69.045 ($C_3H_5N_2$)
Ibuprofen	205.1205	Negative	161.132 ($C_{12}H_{17}$)			
	207.1430	Positive	161.133 ($C_{12}H_{17}$)			
Naproxen	229.0869	Negative	185.095 ($C_{13}H_{13}O$)	124.043 ($C_7H_8O_2$)		
Carbamazepine	237.1020	Positive	210.079 ($C_{13}H_{10}N_2O$)	179.089 ($C_{14}H_{11}$)	128.060 ($C_{10}H_8$)	
Atenolol	267.1696	Positive	249.160 ($C_{14}H_{21}N_2O_2$)	195.090 ($C_{10}H_{13}NO_3$)	145.111 ($C_7H_{15}NO_2$)	135.068 (C_8H_9NO)

Compound	Precursor mass	Ionisation mode	Fragment 1: m/z (formula)	Fragment 2: m/z (formula)	Fragment 3: m/z (formula)	Fragment 4: m/z (formula)
Hydrochloro thiazide	295.9564	Negative	267.934 ($C_6H_5ClN_2O_4S_2$)	204.985 ($C_6H_6ClN_2O_2S$)	202.972 ($C_6H_4ClN_2O_2S$)	
	297.9729	Positive	152.966 ($C_3H_4ClNO_2S$)	146.015 ($C_4H_6N_2O_2S$)	79.980 (H_2NO_2S)	
Codeine	300.1588	Positive	284.165 ($C_{18}H_{22}NO_2$)	194.117 ($C_{11}H_{16}NO_2$)	164.107 ($C_{10}H_{14}NO$)	
Furosemide	329.0010	Negative	285.012 ($C_{11}H_{10}ClN_2O_3S$)	204.982 ($C_6H_6ClN_2O_2S$)		
	331.0157	Positive				

Following on from this, the wastewater aliquots that had been spiked with both modifiers and biomarkers were analysed, as shown in figures 3 and 4, and in table 2. These results show that all the previously detected biomarkers were detected, with at least one fragment each, in either ionisation mode.

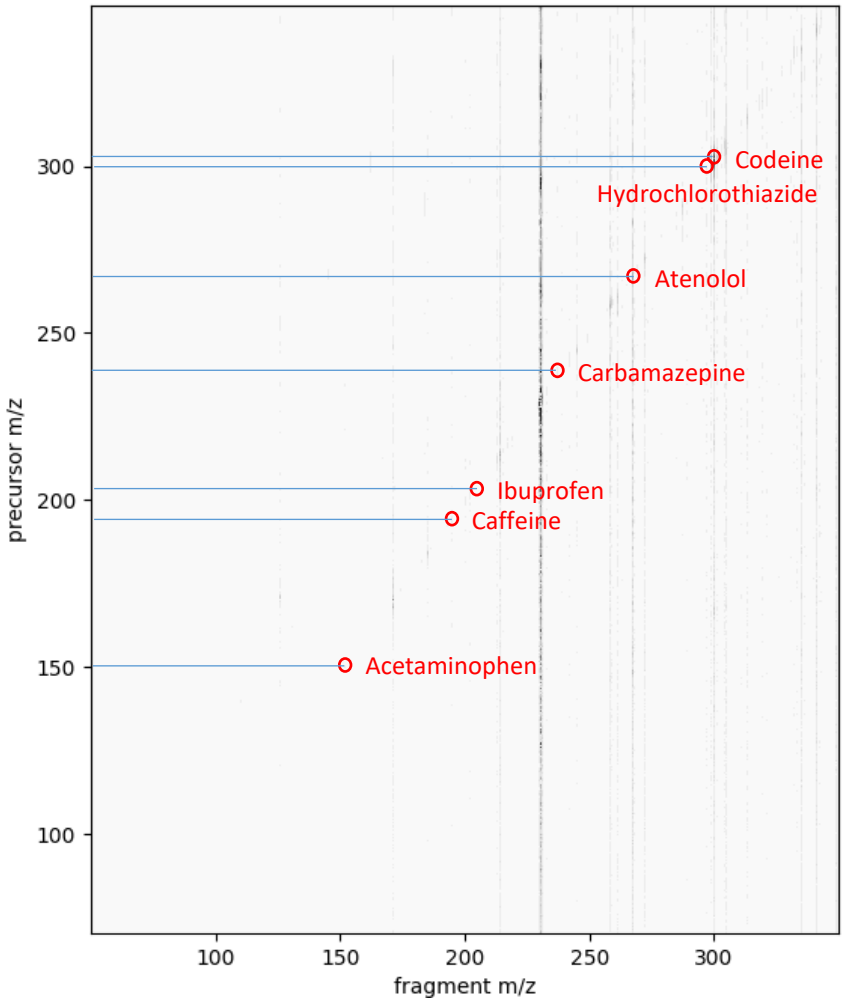

Figure 3. Biomarkers spiked into influent wastewater – ESI+ analysis

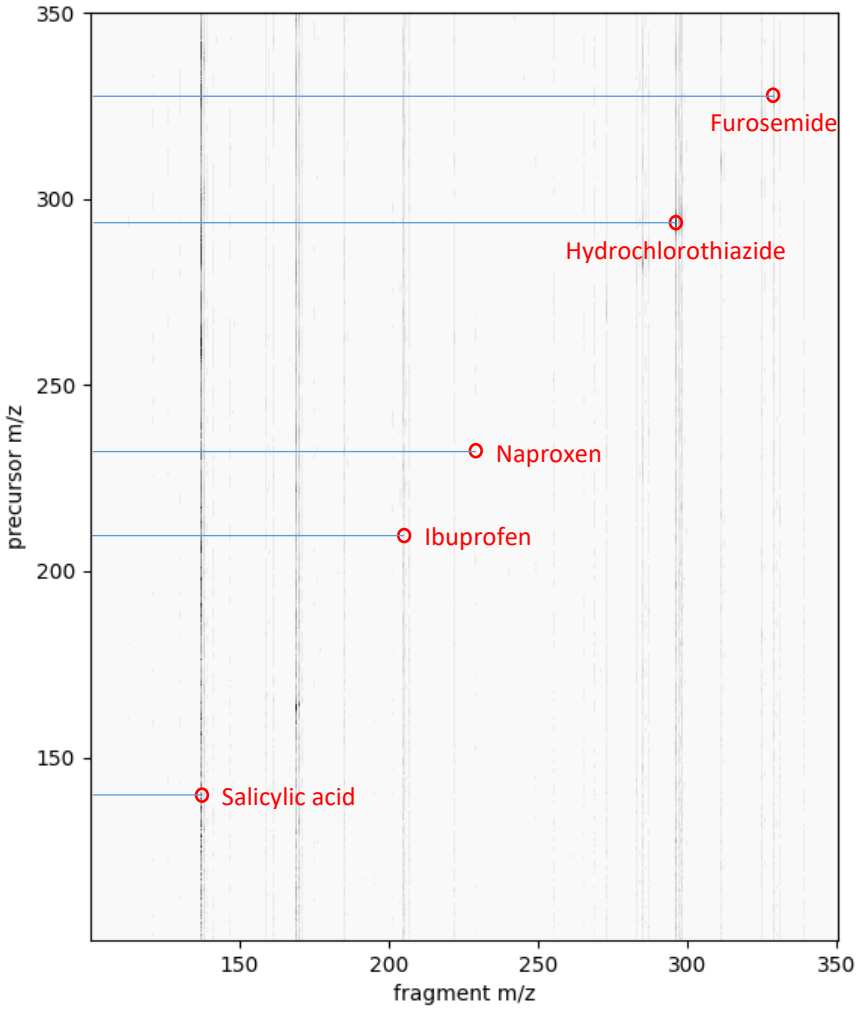

Figure 4. Biomarkers spiked into influent wastewater – ESI- analysis

Table 2. Detected precursors and fragments of population equivalence biomarkers spiked into wastewater

Compound	Precursor mass	Ionisation mode	Fragment 1: m/z (formula)	Fragment 2: m/z (formula)	Fragment 3: m/z (formula)	Fragment 4: m/z (formula)
Salicylic acid	137.0248	Negative	121.038 ($C_7H_5O_2$)	N/D		
Acetaminophen	152.0703	Positive	138.070 ($C_7H_6NO_2$)	N/D	93.033 (C_6H_5O)	N/D
Caffeine	195.0884	Positive	180.066 ($C_7H_8N_4O_2$)	138.066 ($C_6H_8N_3O$)	97.040 ($C_4H_5N_2O$)	N/D
Ibuprofen	205.1205	Negative	161.134 ($C_{12}H_{17}$)			
	207.1387	Positive	N/D			
Naproxen	229.0869	Negative	185.094 ($C_{13}H_{13}O$)	N/D		
Carbamazepine	237.1030	Positive	N/D	179.086 ($C_{14}H_{11}$)	N/D	
Atenolol	267.1710	Positive	249.158 ($C_{14}H_{21}N_2O_2$)	195.089 ($C_{10}H_{13}NO_3$)	145.113 ($C_7H_{15}NO_2$)	135.070 (C_8H_9NO)
Hydrochloro thiazide	295.9564	Negative	267.938 ($C_6H_5ClN_2O_4S_2$)	204.988 ($C_6H_6ClN_2O_2S$)	202.973 ($C_6H_4ClN_2O_2S$)	
	297.9725	Positive	N/D	N/D	N/D	

Compound	Precursor mass	Ionisation mode	Fragment 1: m/z (formula)	Fragment 2: m/z (formula)	Fragment 3: m/z (formula)	Fragment 4: m/z (formula)
Codeine	300.1601	Positive	N/D	194.114 ($C_{11}H_{16}NO_2$)	164.110 ($C_{10}H_{14}NO$)	
Furosemide	329.0010	Negative	285.012 ($C_{11}H_{10}ClN_2O_3S$)	204.981 ($C_6H_6ClN_2O_2S$)		

Finally, wastewater that was only spiked with modifiers (formic acid or ammonium hydroxide) was analysed, in order to detect population biomarkers natively occurring in wastewater (table 3). For each of the biomarkers detected, at least one previously identified fragment was also detected to confirm their identification. Additionally, other biomarkers that have previously been detected in wastewater (4) were also identified, including biomarkers of oxidative stress, and are shown in figures 5 and 6 (blue squares) alongside previously detected population size biomarkers (red circles), and in table 4.

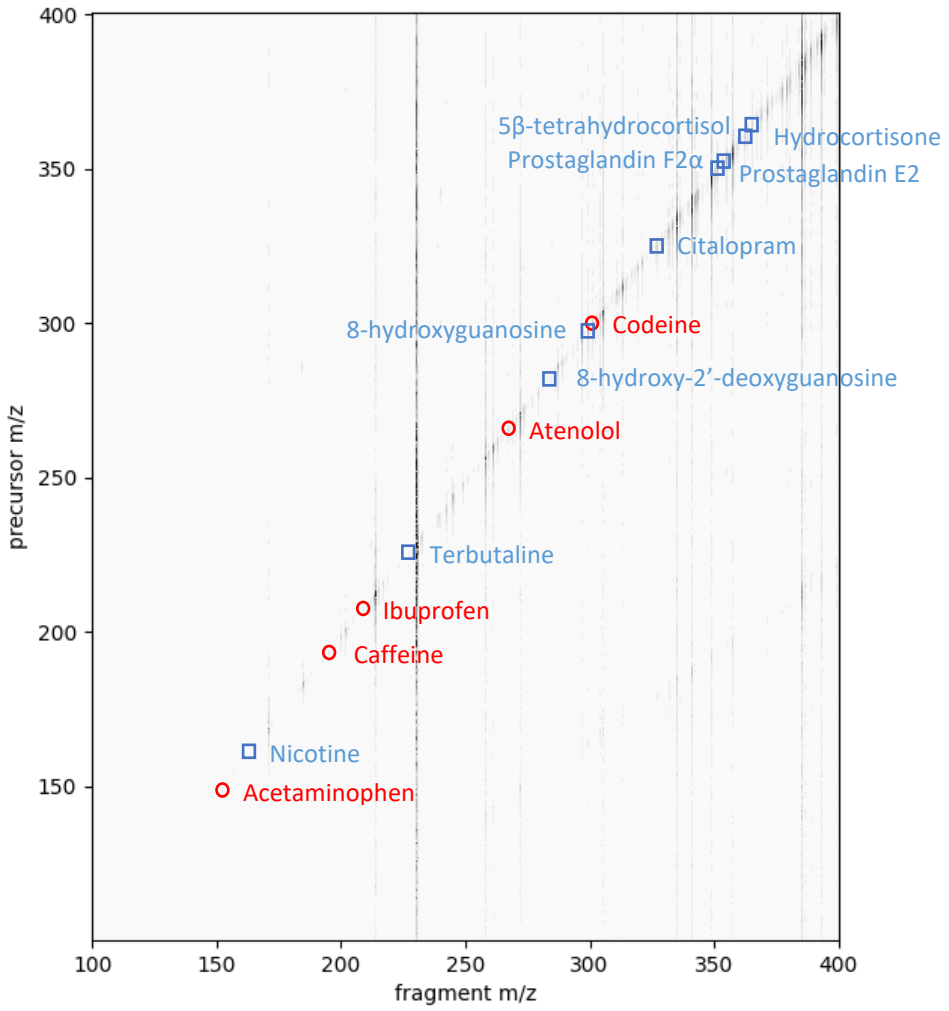

Figure 5. Biomarkers natively identified in influent wastewater – ESI+ analysis

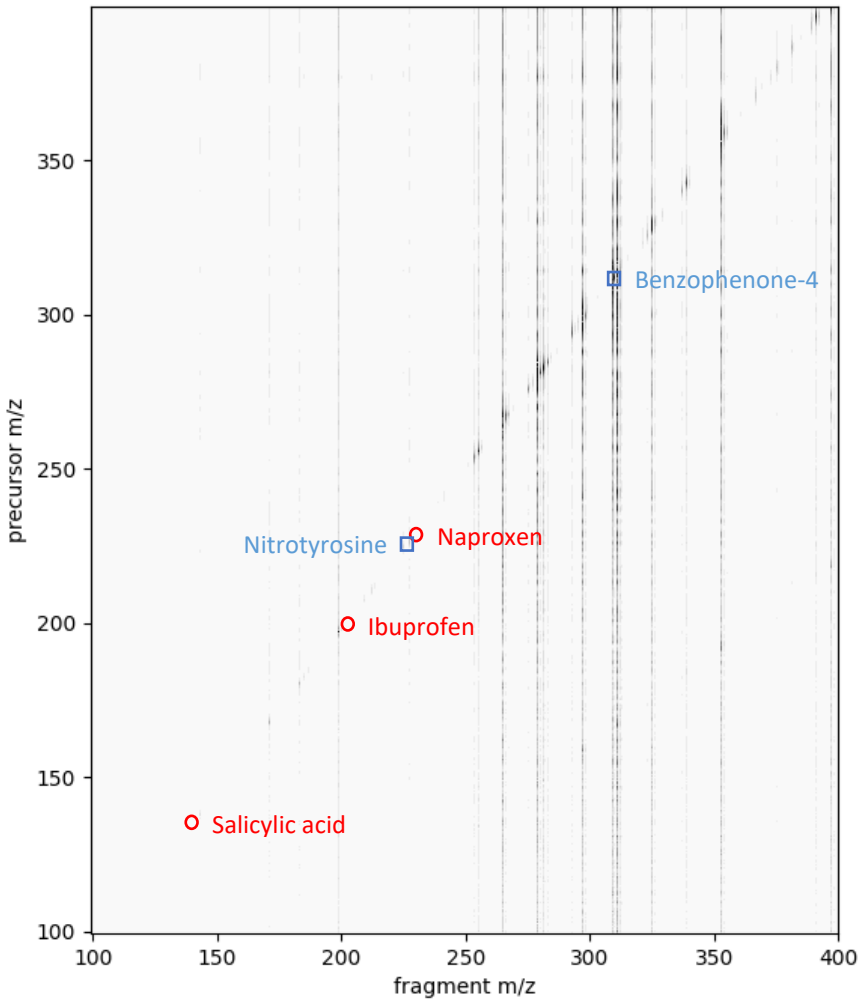

Figure 6. Biomarkers natively identified in influent wastewater – ESI- analysis

Table 3. Precursors and fragments of population equivalence biomarkers detected natively in wastewater

Compound	Precursor mass	Ionisation mode	Fragment 1: m/z (formula)	Fragment 2: m/z (formula)	Fragment 3: m/z (formula)	Fragment 4: m/z (formula)
Salicylic acid	137.0240	Negative	121.038 ($C_7H_5O_2$)	N/D		
Acetaminophen	152.0713	Positive	138.074 ($C_7H_6NO_2$)	N/D	N/D	N/D
Caffeine	195.0884	Positive	180.061 ($C_7H_8N_4O_2$)	N/D	97.039 ($C_4H_5N_2O$)	N/D
Ibuprofen	205.1257	Negative	161.134 ($C_{12}H_{17}$)			
	207.1430	Positive	161.130 ($C_{12}H_{17}$)			
Naproxen	229.0850	Negative	185.089 ($C_{13}H_{13}O$)	124.033 ($C_7H_8O_2$)		
Carbamazepine	N/D	Positive	N/D	N/D	N/D	
Atenolol	267.1710	Positive	249.145 ($C_{14}H_{21}N_2O_2$)	195.091 ($C_{10}H_{13}NO_3$)	145.105 ($C_7H_{15}NO_2$)	N/D
Hydrochloro thiazide	N/D	Negative	N/D	N/D	N/D	
	N/D	Positive	N/D	N/D	N/D	

Compound	Precursor mass	Ionisation mode	Fragment 1: m/z (formula)	Fragment 2: m/z (formula)	Fragment 3: m/z (formula)	Fragment 4: m/z (formula)
Codeine	300.1588	Positive	N/D	194.120 ($C_{11}H_{16}NO_2$)	N/D	
Furosemide	N/D	Negative	N/D	N/D		

Table 4. Potential biomarkers detected through retrospective analysis of wastewater

Compound	Precursor mass	Ionisation mode	Compound	Precursor mass	Ionisation mode
Nicotine	163.1235	Positive	8-Nitroguanine	197.078	Positive
Nitrotyrosine	225.056	Negative	Terbutaline	226.1443	Positive
8-Hydroxy-2'-deoxyguanosine	284.089	Positive	8-Hydroxy guanosine	300.0885	Positive
Citalopram	325.1716	Positive	Prostaglandin E2	353.231	Positive
Prostaglandin F2α	355.241	Positive	Hydrocortisone	363.221	Positive
5β-Tetrahydro cortisol	367.245	Positive			

To confirm the detection of the oxidative stress biomarkers, their ionizability and fragmentation patterns were analysed using pure standards. As shown in figure 7 and table 5, fragments for several biomarkers were identified in influent wastewater and confirmed with standards.

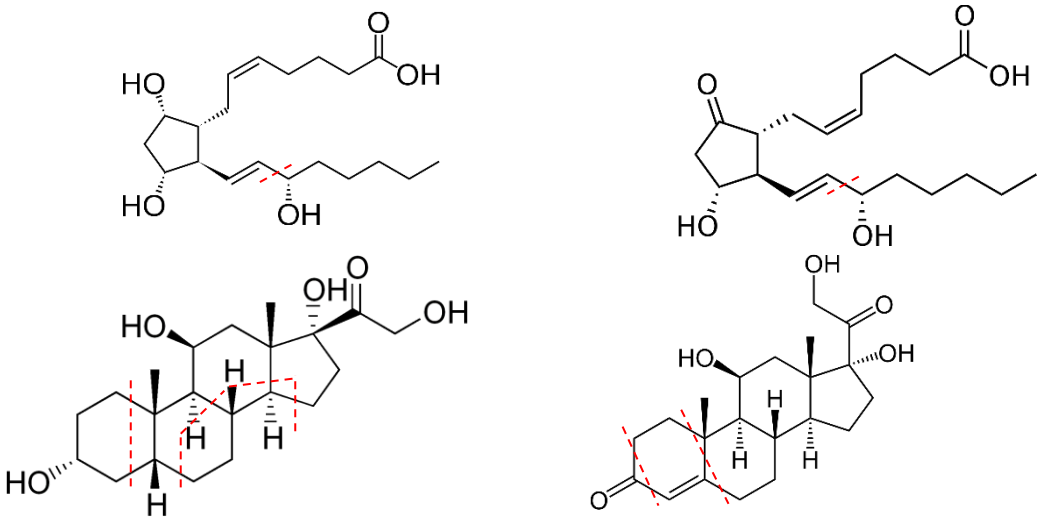

Figure 7. Fragmentation patterns of (top left, clockwise) PF2$_\alpha$, PE2, hydrocortisone and 5β-HYC

Table 5. Precursors and fragments of population equivalence biomarkers detected natively in wastewater

Compound	Precursor mass	Fragment 1: m/z (formula)	Fragment 2: m/z (formula)
Prostaglandin E2	353.231	252.136 ($C_{14}H_{22}O_4$)	
Prostaglandin F2α	355.241	254.149 ($C_{14}H_{24}O_4$)	
Hydrocortisone	363.221	335.220 ($C_{20}H_{31}O_4$)	283.193 ($C_{16}H_{27}O_4$)
5β-Tetrahydrocortisol	367.245	313.203 ($C_{17}H_{29}O_5$)	297.188 ($C_{17}H_{29}O_4$)

Conclusion

Using Total Correlation Mass Spectrometry (TOC-MS) a panel of biomarkers in wastewater could be rapidly identified without complex method development. Additional biomarkers, including pharmaceuticals, lifestyle chemicals and biomarkers of oxidative stress, were then identified through retrospective analysis of previously analysed wastewater data. The ability of TOC-MS to automatically correlate related fragments and precursors simplified the process of identifying these biomarkers by allowing for fragments to be easily identified and then referenced against molecular structure. The novel use of ultraviolet photodissociation (UVPD) to fragment small molecules allowed access to more structural diverse fragmentation patterns, which can further assist in identifying unknown analytes in a complex matrix, such as wastewater.

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