Identification of biomarkers of population size in wastewater using Total Correlation Mass Spectrometry (ToC-MS)

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Wastewater-based epidemiology

Wastewater based epidemiology (WBE) is a technique that seeks to understand public health through analysis of wastewater as a surrogate, community-wide pooled urine sample [1]. First posited in 2001 [2], WBE has since gone on to see widespread adoption for its ability to monitor illicit drugs [3], compounds of environmental concern, such as pesticides [4], and pharmaceuticals [5] as well as more recent applications, such as monitoring general public health [6] or specific diseases within a population [7], or on improving the quality of WBE results [8]. The shift towards analysing general public health using WBE was important as it allowed for the assessment of the whole population for the first time, using novel biomarker targets such as biomarkers of oxidative stress [6,9]. The advantage of WBE is that it allows for the use of a single, anonymous sample to assess public health.

Figure 1: Flowchart of wastewater-based epidemiology process

Back calculation for WBE

When analysing either general or specific health biomarkers with WBE there is an important need to standardise the results such that they can be compared both spatially and temporally [10], taking into account the amount of wastewater passing through the sewer or treatment works over a given period and the population contributing to it. The amount of wastewater passing through a treatment works in a day is often a known variable, and as such is easy to correct. However, population is a more dynamic variable and is often determined using static references, such as population censuses, or estimates based on the number and size of houses in an area.

To overcome this, a number of approaches have been trialed to use biomarkers within wastewater to calculate population size; effectively allowing a sample to be normalised by itself. The most extensive of these approaches was carried out in Australia on the day of the 2016 census [8], i.e. when population estimates would be at their most accurate. This study used LC-MS/MS and Bayesian to successfully identified a number of small molecule, chemical biomarkers in wastewater that could be used to independently calculate population size.

Total Correlation Mass Spectrometry (ToC-MS)

Total Correlation Mass Spectrometry (ToC-MS) is a new MS analytical technique that aims to provide DIA 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 [11] (2D-MS), which controls how molecules fragment such that the fragments can be intrinsically linked to their original molecules without first separating them. For more details please see our talk Tuesday Afternoon (**TOB PM**) or visit us at **Booth 314**.

For WBE, ToC-MS can allow users to effectively analyse wastewater without having to target specific molecules, and risk missing crucial information, or worry about chimeric spectra. The ToC-MS platform allows also removes the need for LC to separate analytes such that only a few enter the instrument at any one time, which decreases the amount of method development required and increases the speed of analysis to support near real time analysis of wastewater.

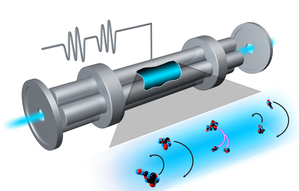
Image 1: Overview of total correlation MS procedure

Methods and materials

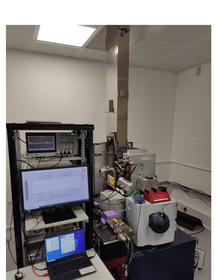
Image 2: image of current ToC-MS instrument

The aim of the project was to identify a panel of previously identified population equivalence biomarkers (PEBs) using ToC-MS and then extend the analysis to biomarkers of oxidative stress. All analytes, solvents and reagents were purchased from Sigma Aldrich (Gillingham, UK) or Cambridge Bioscience (Cambridge, UK (italicised)) and were of the highest grade possible. The selected biomarkers included a number of pharmaceuticals and lifestyle biomarkers: acetaminophen, atenolol, caffeine, carbamazepine, codeine, furosemide, gabapentin, hydrochlorothiazide, ibuprofen, naproxen and salicylic acid. Biomarkers of oxidative stress included prostaglandins E2 and F2α, 8-hydroxy-2′-deoxyguanosine (8-OH-dG), cortisol, 5-hydroxyindole-3-acetic acid (HIAA), *5β-tetrahydrocortisone*, *8-hydroxyguanosine*, *8-nitroguanine*, *4-hydroxy nonenal mercapturic acid* and *nitrotyrosine*.

All ToC-MS analyses were carried out by direct infusion of sample into the instrument at a flow rate of 2.5 uL min^{-1} and fragmented using an eMOPA213-20, 213 nm laser from CryLas. Initially, biomarkers were analysed in methanol with 0.1% formic acid (v/v) at a concentration of 100 ng mL^{-1} in order to identify their UVPD fragmentation patterns. Biomarkers were then spiked into 100 mL of influent wastewater that had been extracted using Waters Oasis HLB cartridges and eluted using 4 mL of methanol containing 0.1% (v/v) formic acid. Finally, natively occurring biomarkers were identified in a separate aliquot of influent wastewater that had been extracted in the same way but to which no biomarkers were added.

Population equivalence biomarkers

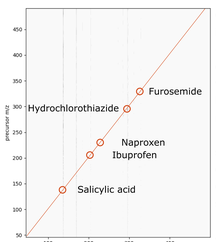

Figure 2: Negative ESI analysis of standards in wastewater

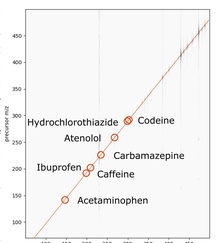

Figure 3: Positive ESI analysis of standards in wastewater

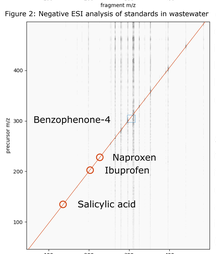

Figure 4: Negative ESI analysis of influent wastewater

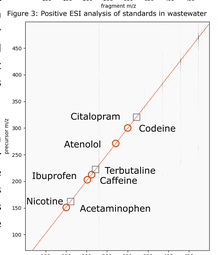

Figure 5: Positive ESI analysis of influent wastewater

Of the eleven selected biomarkers that were spiked into extracted influent wastewater, ten were identified via the ToC-MS process (figures 2 and 3). The one outlier, gabapentin, was likely not detected as it lacked a suitable chromophore for UVPD and as such would not appear in the final ToC-MS plot. Ibuprofen and hydrochlorothiazide could be identified using either positive or negative ESI, but both had greater signal intensity under negative ESI conditions. For each of the successful biomarkers a number of structurally confirmed fragments were identified, ranging between one and four depending on the molecule. Other molecular signals could be observed in the ToC-MS plot but were not identified at this stage.

When observing natively occurring biomarkers in wastewater, eight of our ten previously identified biomarkers were detected in extracted influent wastewater and confirmed using their previously identified fragmentation patterns. Carbamazepine and hydrochlorothiazide were potentially not detected due to insufficient loads of the substance in wastewater on the day the sample was collected.

A number of other small molecule biomarkers were then identified in the ToC-MS plot, using a previous publication examining wastewater from the same site as a guide for what biomarkers could be expected [12] (figures 4 and 5). An initial cursory examination was able identify a number of other potential population biomarkers including nicotine, citalopram (anti-depressant), terbutaline (bronchodilator) and benzophenone-4 (UV filter).

The power of ToC-MS comes from it's ability to intrinsically correlate fragments and precursors without relying on prior quadrupolar isolation or chromatographic separation. Figure 6 shows an extracted chromatogram for atenolol in wastewater, analogous to that produce from isolation and fragmentation of atenolol using a Q-ToF. This figure shows the identified fragmentation pattern of atenolol produced from UVPD. By controlling how molecules are fragmented within the ToC-MS instrument by modulating their position relative to the UVPD beam ToC-MS is able to prove that two ions are related as they'll have the same frequency of modulation. This is shown in figures 7 and 8 where atenolol fragment 4 (figure 7) has the same frequency of modulation as the parent atenolol molecule (figure 8).

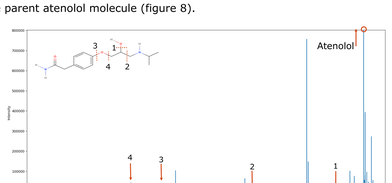

Figure 6: Horizontal extracted chromatogram of atenolol, atenolol intensity is off the scale of this image

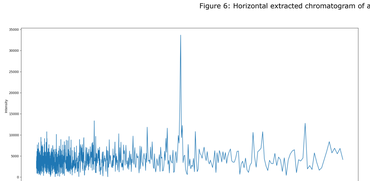

Figure 7: Vertical extracted chromatogram of atenolol fragment 4

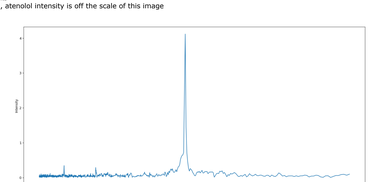

Figure 8: Vertical extracted chromatogram of atenolol (scale is e6)

Retrospective analysis of biomarkers of oxidative stress

As a data independent analytical technique ToC-MS is eminently suitable for retrospective analysis, as demonstrated in the previous section by the detection of citalopram, nicotine, terbutaline and benzophenone-4. This approach was repeated to look at a number of biomarkers of oxidative stress that have previously been identified in wastewater [7,9]. Figure 9 shows that a number of biomarkers of oxidative stress could be identified through retrospective data mining of the influent wastewater sample shown in figure 5. Of these the most abundant were prostaglandin E2(PE2) and F2α (PF2α), cortisol and its metabolite 5β-tetrahydrocortisol (5β-THC). Retrospective analysis of the same wastewater with negative ESI conditions, as shown in figure 4, only yielded nitrotyrosine (not shown here).

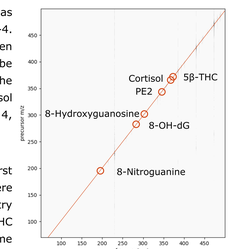

Figure 9: Retrospective ESI⁻ analysis of influent wastewater

In order to positively identify these analytes in wastewater their UVPD fragmentation patterns were first identified by analysis of analytical standards using direct infusion ToC-MS. The identified fragments were confirmed by accurate mass and comparison to the structure of the parent molecule, and were then used to try and identify the parent molecule in wastewater. In this manner fragments of PE2, PF2α, cortisol and 5β-THC were identified in wastewater. For the prostaglandins, there was one fragment identified for each at the same relative position between the carbon-carbon double bond and alcohol on the allylic substituent of the prostaglandin ring (figures 10 and 11). For cortisol and 5β-THC two fragments were identified, with the majority of fragments centered around the steroid core rather than the alkyl substituents (figures 12 and 13).

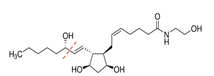
Figure 10: UVPD fragmentation of PF2α

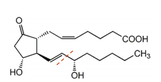
Figure 11: UVPD fragmentation of PE2

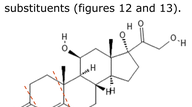
Figure 12: UVPD fragmentation of cortisol

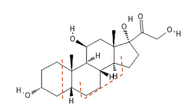
Figure 13: UVPD fragmentation of 5β-THC

Other applications for ToC-MS

Lipidomics - Fragmentation at C=C bonds, as shown for the prostaglandins can be used to locate double bond position, as well as lipid tail and headgroup, in lipids without requiring analyte derivatisation or MS^3 fragmentation (See our application note for more details!)

Multi-omics - Integration of multiple sample types in a single analysis with high reproducibility and elevated sensitivity

Complex samples - ToC-MS increases the number of analytes detected and simultaneously improves data quality by providing MS/MS data for every precursor and enabling retrospective data analysis.

Conclusions

Using Total Correlation Mass Spectrometry (ToC-MS) a method was developed that allowed for the identification of a panel of potential population equivalence biomarkers in wastewater. Additional biomarkers, including pharmaceuticals, lifestyle chemicals and biomarkers of oxidate stress, were then identified through retrospective analysis of the already 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.

References

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