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Enantioselective LC-MS/MS for anthropogenic markers of septic tank discharge

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Abstract

Households in rural locations utilize septic tanks for wastewater treatment and can cause surface water contamination. A new methodology was developed to help investigate the role septic tanks play in the dissemination of prescription and over-the-counter drugs, personal care products and stimulants in the aqueous environment. Simultaneous analysis of 16 chiral and achiral anthropogenic markers was achieved using a Chirobiotic V2® enantioselective column in polar ionic mode. The optimized method achieved quantitation limits for 16 compounds in the range 0.001-2.9 µg L^{-1} and 0.0002-0.43 µg L^{-1} for septic tank effluent and stream water, respectively. Application of the method to samples collected in North East Scotland found caffeine to be ubiquitous in all samples studied suggesting it as a good indicator of septic tank discharge. In rural streams studied, concentrations of all prescription drugs investigated were ≤0.02 µg L^{-1}. However, analgesics and stimulants were at high concentration in one location indicating direct discharge of septic tank wastewater (i.e., not dissipated through a soak away). For example, paracetamol, cotinine and caffeine were measured at 1,100 µg L^{-1}, 31 µg L^{-1} and 200 µg L^{-1}, respectively, which is comparable to septic tank effluents. Furthermore, *S(+)*-amphetamine and *R(-)*-amphetamine were present in this stream sample at 0.20 and 0.27 µg L^{-1}. This corresponds to an enantiomeric fraction of 0.43, which is typical of untreated wastewaters in the UK. Findings illustrate further study on the diffuse impact of septic tanks to surface water is needed and can be supported using this new multi-residue enantioselective method.

Keywords: septic tank; rural; pharmaceutical; chiral; wastewater; mass spectrometry

1. Introduction

Anthropogenic chemicals such as pharmaceuticals and personal care products are ubiquitous in surface waters receiving municipal wastewater discharges (Hughes et al., 2013; Petrie et al., 2015). The presence of anthropogenic chemicals in surface waters is concerning due to their pharmacological active nature and the possible detrimental impact to aquatic organisms (Kasprzyk-Hordern, 2010; Hughes et al., 2013). The majority of research to date has focused on the impact of effluent discharges from communal wastewater treatment plants (WWTPs) (Nakada et al., 2006; Gardner et al., 2012; Baker and Kasprzyk-Hordern et al., 2013; Archer et al., 2017). However, a notable portion of the population can be served by onsite wastewater treatment processes such as septic tanks. It is estimated that such systems (or similar) serve 20 % of households in the United States (Schaider et al., 2017) and 33 % in Ireland (Carlow Tanks, 2018). In Scotland, there are 161,000 known private wastewater discharges (CREW, 2018). Assuming an average number of inhabitants per household of 2.16 (National Records of Scotland, 2018), this would equate to a conservative estimate of 7 % of the Scottish population using a septic tank.

Septic tank systems consist of a concrete or plastic chamber which allows settling of solids and flotation of fat, oil and grease. It is considered that wastewater needs retained within the tank for a minimum of 24 h to pass through the system at slow velocity and turbulence for treatment (Seabloom et al., 2005). The anaerobic environment facilitates slow growing bacteria which decompose organic matter. However, solids enter the tank at a faster rate than they are broken down. Therefore it is recommended that septic tanks need emptied every 1-2 years (Carlow Tanks, 2018). The quality of septic tank effluent is considerably poorer than that of conventional (aerobic) communal WWTPs such as trickling filters. For further treatment the effluent typically enters a soak away/septic drain field and is dissipated in the environment (Schaider et al., 2017). This can lead to the contamination of ground water and surface water with anthropogenic chemicals such as pharmaceuticals (Schaider et al., 2017). The potential for contamination of water bodies by septic systems can be increased by poor tank maintenance. Furthermore, septic tanks are often historical systems with little knowledge on their configuration or maintenance history.

Septic tanks are not designed for the removal of trace contaminants. Consequently, effluents from septic tanks have previously been found to contain prescription drugs, over-the-counter drugs, stimulants, personal care products and their metabolites (Hinkle et al., 2005; Carrara et al., 2008; Conn et al., 2010; Phillips et al., 2015; Schaider et al., 2017). Such compounds (which do not have veterinary uses) are useful indicators of septic tank discharge entering both ground and surface waters. In septic tank effluent these markers vary in concentration from a few ng L^{-1} to mg L^{-1} and their fate and removal in drain fields can vary greatly (Schaider et al., 2017). The majority of research to date has focused on the influence of septic tank discharges to ground water and drinking water quality (Hinkle et al., 2005; Swartz et al., 2006; Godfrey et al., 2007; Phillips et al., 2015; Schaider et al., 2016). However, septic tanks can be located close to small streams which form sub-catchments of larger rivers. These small streams are themselves important ecosystems and can be used to help estimate the contribution of septic tanks to riverine concentrations of anthropogenic chemicals. Nevertheless, information on the impact of septic tanks to rural surface water quality is scarce.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is preferred for analysis of pharmaceuticals and related chemicals in the environment due to its excellent sensitivity and specificity. It is recommended that analysis of chiral anthropogenic chemicals is undertaken at the enantiomeric level (Kasprzyk-Hordern, 2010; Sanganyado et al., 2017). This is essential for risk assessment due to enantiospecific toxicity of chiral species (Stanley et al., 2006; 2007; De Andrés et al., 2009). For example, *R(-)*-fluoxetine is approximately 30 times more toxic than *S(+)*-fluoxetine towards *Tetrahymena thermophila* (De Andrés et al., 2009). Furthermore, investigating the enantiomeric distribution of chiral analytes helps understand their source, fate and transport in the water cycle (Bagnall et al., 2013; Emke et al., 2014; Petrie et al., 2016a). This is because chiral analytes can undergo (varying degrees of) stereoselective metabolism within the human body, during wastewater treatment and in the environment itself. Nevertheless, there is a general lack of enantioselective methods in the literature for environmental analysis. It is important that enantioselective methods support the simultaneous determination of achiral anthropogenic markers for a holistic understanding of water quality with respect to these chemicals. Existing methods that

measure anthropogenic chemicals in wastewaters and surface waters do not support multi-residue enantioseparations and achiral analyte determinations (Bagnall et al., 2012; Lopez-Serna et al., 2013; Zhao et al., 2016) or have chromatographic run times (e.g., >60 min) (Lopez-Serna et al., 2013; Camacho-Muñoz and Kasprzyk-Hordern, 2015; Camacho-Muñoz and Kasprzyk-Hordern, 2017).

Therefore, the aim of the study was to develop a new analytical methodology (including sample storage, extraction and instrumental analysis) for the multi-residue determination of chiral and achiral anthropogenic markers of septic tank discharge in a run time <60 min. A total of 16 anthropogenic markers (over-the-counter medication, prescription drugs, stimulants and personal care products) were analysed simultaneously by LC-MS/MS using a Chirobiotic V2® enantioselective column. The developed method was applied to septic tank effluents and surface waters in North East Scotland.

2. Materials and methods

2.1. Materials

The analytical standards aspartame, methylparaben, triclocarban, caffeine, carbamazepine, carbamazepine 10,11 epoxide, cotinine, paracetamol, *R/S(±)*-amphetamine, *R/S(±)*-atenolol, *R/S(±)*-chlorpheniramine, *R/S(±)*-citalopram, *R/S(±)*-fluoxetine, *R/S(±)*-MDMA, *R/S(±)*-propranolol and *R/S(±)*-salbutamol were purchased from Sigma-Aldrich (Gillingham, UK) as well as the following labelled surrogate standards: caffeine-13C3, carbamazepine-d10, carbamazepine 10,11 epoxide-d10, cotinine-d3, paracetamol-d4, triclocarban-d3, *R/S(±)*-amphetamine-d11, *R/S(±)*-atenolol-d7, *R/S(±)*-chlorpheniramine-d6, *R/S(±)*-citalopram-d6, *R/S(±)*-fluoxetine-d6, *R/S(±)*-MDMA-d5, *R/S(±)*-propranolol-d7 and *R/S(±)*-salbutamol-d3. Oasis HLB (60mg, 3mL) cartridges for solid phase extraction (SPE) were obtained from Waters (Manchester, UK). HPLC-grade methanol, ammonium acetate and acetic acid were purchased from Fisher Scientific (Loughborough, UK). Ultra-pure water used throughout the study was of 18.2 MΩ cm^{-1} quality. For method development and validation, effluent (5 L) was collected from a septic tank which serves 7 inhabitants in Aberdeenshire, North East Scotland. Stream water (10 L) was collected from a tributary of the River Don, Aberdeenshire.

2.2. Sample collection and solid phase extraction

All samples were collected (1 L for septic tank effluent and surface water) and transported in polypropylene bottles (Petrie et al., 2017). These were kept dark and cooled to 4 ˚C whilst transported to the laboratory for processing. Firstly, septic tank effluent and stream samples were filtered through 0.7 µm GF/F filters (Fisher Scientific). Aliquots of 25 mL effluent and 250 mL stream water were then spiked with 100 ng of all deuterated surrogates (100 µL of a 1,000 µg L^{-1} methanolic mixture). For SPE, Oasis HLB cartridges were conditioned with 2 mL methanol and equilibrated with 2 mL water under gravity at a rate of 1 mL min^{-1}. Effluent and stream water were then loaded at 5 mL min^{-1}, washed with 10 mL water and dried. 4 mL methanol was subsequently used to elute analytes under gravity at 1 mL min^{-1} which were accordingly dried using nitrogen stream at 40 °C. Dried residues were reconstituted in 250 µL mobile phase (methanol containing 1 mM ammonium acetate and 0.01 % acetic acid) and filtered through 0.2 µm LC-MS pre-filters ready for

enantioselective LC-MS/MS analysis. All samples were prepared in triplicate and analysed within 24 h of collection. Prepared samples containing anthropogenic markers above their respective calibration ranges were appropriately diluted and re-analyzed.

2.3. Enantioselective LC-MS/MS

An Agilent 1200 Infinity Series HPLC coupled to a 6420 MS/MS triple quadrupole (Cheshire, UK) was used for analysis. Separation was performed using a Chirobiotic V2® HPLC column (250 x 2.1 mm; 5 µm) maintained at 15 °C. The final mobile phase was methanol containing 1 mM ammonium acetate and 0.01 % acetic acid. This was operated under isocratic conditions with a flow rate of 0.17 mL min^{-1}. The injection volume was 40 µL and run time 55 min.

Electrospray ionisation (ESI) in both positive and negative modes with a capillary voltage of 4,000 V was used. Nitrogen was the nebulising, desolvation and collision gas. The desolvation temperature was 350 °C with a gas flow of 12 L min^{-1}. The nebulizing pressure was 50 psi. All analytes were analysed in positive mode except methylparaben, triclocarban and triclocarban-d3 which were analysed in negative mode. Optimized multiple reaction monitoring (MRM) transitions for each analyte are compiled in Table S1.

2.4. Instrument and method performance

A 13-point calibration curve ranging in concentration from 0 to 5,000 µg L^{-1} was used to establish linearity. For chiral analytes this represents their total enantiomeric concentration (i.e., 5,000 µg L^{-1} is equivalent to 2,500 µg L^{-1} of each enantiomer). To determine intra- and inter-day precision and accuracy, triplicate injections of 10, 100 and 500 µg L^{-1} standards were, respectively, conducted within 24 h and over 3 different days. Instrument detection limits (IDLs) were determined by the lowest concentration at which the signal-to-noise ratio (S/N) ≥3 and instrument quantitation limit (IQL) when S/N ≥10. Sensitivity of the SPE-enantioselective LC-MS/MS method was determined by calculating the method detection limit (MDL) and method quantitation limit (MQL) for each analyte:

$$MDL\ (\mu g\ L^{-1}) = \frac{IDL\ x\ 100}{Rec\ x\ CF} \quad [1]$$

$$MQL\ (\mu g\ L^{-1}) = \frac{IQL\ x\ 100}{Rec\ x\ CF} \quad [2]$$

Here *IDL* and *IQL* are the instrumental detection and quantitation limits, respectively (µg L^{-1}), *Rec* is the absolute analyte recovery (%) and *CF* is the pre-concentration factor (100 for effluent and 1,000 for stream water).

During the development stages the optimum concentration factor for SPE was determined for both septic tank effluent and stream water samples. This involved spiking filtered effluent and stream water with an additional 1 µg L^{-1} of each anthropogenic marker. Concentration factors investigated were 25, 50, 100, 250 and 500 for effluent and 100, 250, 500, 1,000 and 2,000 for stream water.

Method recovery was established by spiking filtered environmental samples at two concentration levels. Effluent was spiked at 0.5 µg L^{-1} and 5 µg L^{-1} whereas stream water was spiked at 0.05 and 0.5 µg L^{-1}. Signal suppression caused by co-extracted matrix was assessed by extracting samples as described previously and spiking SPE extracts to achieve a final theoretical concentration of 200 µg L^{-1}. The suppression of analyte signal intensity using the developed SPE method was quantified using the following equation:

$$Signal\ suppression\ (\%) = 100 - \left(\frac{(A\ spiked\ extract - A\ unspiked\ extract)}{A\ standard}\ x\ 100\right) \quad [3]$$

Where *A spiked extract* is the peak area of analyte in extracts spiked post-SPE, *A unspiked extract* is the peak area of analyte in extracts not spiked and *A standard* is the peak area of analyte in a standard solution which corresponds to the spike. All analysis was performed in triplicate.

2.5. Anthropogenic marker stability in collected samples

The stability of analytes was assessed under typical sample transport/storage conditions. Both freshly collected septic tank effluent and stream water were spiked to ensure adequate levels of all anthropogenic markers for detection (5 µg L^{-1} and 0.5 µg L^{-1}, respectively), and mixed. Sample volumes of 4 L were prepared in polypropylene bottles and stored in the dark at both room temperature (18 ±0.5 ˚C) and 4 ±0.5 ˚C (Petrie et al., 2017). Bottles were then left unmixed to replicate proposed storage conditions. Samples were then taken for analysis and subject to SPE as

described previously at 0, 6, 24 and 48 h. The enantiomeric composition (and changes) of chiral markers can be expressed as enantiomeric fraction (EF) using:

$$EF = \frac{(+)}{[(+)+(-)]} \quad [4]$$

Here *(+)* is the concentration of the *(+)*-enantiomer and *(-)* is the concentration of the *(-)*-enantiomer.

2.6. Profiling anthropogenic markers in septic tank effluents and surface waters

Two sub-catchments of the River Don, Aberdeenshire were investigated (Figure 1). These were studied as they are rural areas without communal wastewater discharges within their catchment area. The land use of both catchments is arable farmland. Any wastewater discharges here are from septic tanks or farmyards. Sub-catchment A contains ~10 septic tanks (estimated population of 30 inhabitants) and a small stream (discharge <0.1 m^3 s^{-1}). Sub-catchment B (Figure 1) contains >100 septic tanks with a population of ~500 inhabitants and a stream with an estimated discharge of ~0.1 m^3 s^{-1}. Permission was granted to sample effluent from 15 septic tanks (Figure 1). All septic tanks were constructed of concrete serving 2-7 inhabitants per tank. A total of 11 stream water samples were collected from sub-catchments A and B. The River Don is impacted by communal wastewater discharges as well as effluent from septic tanks and farmyards. River water was collected upstream and downstream of each sub-catchment location (Figure 1), and at the time of sampling the river discharge was 9.5 m^3 s^{-1}. The nearest communal WWTP discharge is 7 km upstream of sampling point 1 (Figure 1). Sampling was conducted on 21st June 2018.

3. Results and discussion

3.1. Instrumental development and performance

A Chirobiotic V2® enantioselective column was operated in polar ionic mode due to its separation ability for a range of chiral anthropogenic markers at the enantiomeric level including beta-blockers, beta-agonists, anti-depressants, stimulants and anti-histamines. The mobile phase consisted of 1 mM ammonium acetate in methanol containing 0.01 % acetic acid maintained at 0.17 mL min^{-1}. It was found that ammonium acetate concentration and column temperature had the greatest influence on enantioseparations. Reduced mobile phase concentrations of ammonium acetate improved enantioresolution (R_S), however this can lead to reduction in ionization and MS/MS sensitivity for some analytes. The final method utilized a concentration of 1 mM ammonium acetate which gave the best trade-off between R_S and sensitivity for the analytes studied.

Reducing column temperature improved enantiomer separation for the majority of chiral analytes. This is in agreement with Sanganyado et al (2014) who noted that reducing column temperature from 40 ˚C to 13 ˚C improved R_S of both atenolol and fluoxetine enantiomers under similar mobile phase conditions. In our study the column temperature was maintained at 15 ˚C which facilitated satisfactory multi-residue enantiomeric separation within a run time of 55 min. R_S was ≥1 for all chiral anthropogenic markers which showed separation (atenolol, propranolol, salbutamol, fluoxetine, citalopram, amphetamine and chlorpheniramine) (Figure 2). This satisfies a maximum 2 % peak overlap required for quantitative analysis (Bagnall et al., 2012). Under these conditions achiral analytes (caffeine, paracetamol, etc) were also determined. Achiral analytes exhibited retention times between 5 and 10 min due to comparatively less interaction with the chiral vancomycin stationary phase (Figure 2). Nevertheless, peak shape was satisfactory avoiding the need for a separate non-chiral analytical method to encompass a full suite of anthropogenic markers.

Instrument performance for all chiral and achiral analytes was evaluated by investigating linearity, sensitivity and intra- and inter-day precision and accuracy. The majority of analytes exhibited linearity from their respective IQL to 1,000 or 2,500 µg L^{-1} with coefficient of determination (r^2) ≥0.999 (Table S2). IDLs were in the range 0.02-1.5 µg L^{-1} and IQLs 0.05-10 µg L^{-1}. Only aspartame

was out with these ranges due to broad peak shape. Intra- and inter-day precision was generally <5 % whereas accuracy was normally ±10 % for each concentration level studied (Table S3). The instrument performance was similar to previously reported enantioselective vancomycin methods operated in polar ionic mode by LC-MS/MS for both chiral analytes (López-Serna et al., 2013; Evans et al., 2015; Petrie et al., 2018) and achiral analytes (Petrie et al., 2018).

3.2. Extraction and method performance

Oasis HLB cartridges were selected for SPE as they are favoured for multi-residue analysis due to the mixed mode ion exchange and reversed phase retention mechanisms of the co-polymer. Furthermore, extracted samples do not require elution with any additive (e.g., ammonium hydroxide) which can be detrimental to enantioselective separation on vancomycin stationary phases (Evans et al., 2015; Petrie et al., 2018). However, a drawback of using non-selective SPE is the comparatively high concentration of co-extractives in environmental samples containing the analyte of interest. This can lead to severe quenching (or complete loss) of analyte signal strength during ESI (Gros et al., 2006). Extracting more analyte at greater sample pre-concentration factors may not be translated into increased instrument response. A breakthrough can be reached where signal suppression outweighs the advantages of extracting a greater quantity of analyte (as well as sorbent saturation). Therefore, it is essential to investigate the sample pre-concentration factor which gives the highest analyte response, especially when conducting environmental trace analysis.

For septic tank effluent, pre-concentration factors of 25, 50, 100, 250 and 500 were investigated. It was found that analyte response increased proportionally with concentration factors up to 100 (Figure S1). Above this value, response did not increase for some analytes (particularly those with retention times <30 min) and loss of chiral recognition was observed. Therefore, the pre-concentration factor selected for effluent was 100. In stream water analyte response increased linearly over the studied range of pre-concentration factors investigated (100-2,000) (Figure S1). However, at a concentration factor of 2,000 some loss of chiral recognition was found for several analytes, thus a pre-concentration factor of 1,000 was selected for stream water.

Signal suppression during ESI was in the range 20-98 % and 7-96 % for septic tank effluent and stream water, respectively (Table 1). Highest suppression was observed for those analytes with the least interaction with the chiral stationary phase (i.e., shortest retention time). For example, all analytes with a retention time <10 min (methylparaben, paracetamol, carbamazepine, carbamazepine 10,11 epoxide, triclocarban, caffeine and cotinine) exhibited suppression of ≥67 %. On the other hand *R(-)*-fluoxetine, *R(-)*-citalopram, *S(+)*-citalopram, *S(+)*-chlorpheniramine, *R(-)*-chlorpheniramine all had retention times >40 min and suppression was ≤40 % (Table 1). Such levels of signal suppression are typical for enantioselective LC-MS/MS methods for environmental analysis (Bagnall et al., 2012; Lopez-Serna et al., 2013; Camacho-Muñoz and Kasprzyk-Hordern, 2015). It is also important to note that signal suppression between enantiomers of the same chiral marker can vary substantially. To demonstrate, signal suppression of *S(+)*-fluoxetine in stream water was 70 ± 4 % whereas *R(-)*-fluoxetine had suppression of 38 ± 6 % (Table 1), highlighting the necessity of incorporating labelled surrogates for quantitative analysis at the enantiomeric level.

Performance of the overall SPE-enantioselective LC-MS/MS methodology was evaluated by spiking septic tank effluent and stream water at two concentration levels (i.e., 0.5 and 5 μg L^{-1} for septic tank effluent and 0.05 and 0.5 μg L^{-1} for surface water). Absolute recovery (i.e., only taking into account analyte peak area) ranged from 2 % to close to 100 % (Table 1). Corrected recovery or method accuracy which accounts for the deuterated surrogate response was 90-110 % with RSDs <10 % for the majority of analytes studied. However, both methylparaben and aspartame were out with this range. As they were quantified using an alternative deuterated surrogate (caffeine-13C3 and *S(+)*-fluoxetine-d6, respectively), their analysis can only be considered semi-quantitative.

Septic tank effluent MDLs ranged from <0.001 μg L^{-1} to ~1 μg L^{-1} whilst MQLs up to ~3 μg L^{-1} were determined (Table 1). In stream water MDLs and MQLs were approximately 10 times lower due to the cleaner matrix and greater sample pre-concentration that were applied. MDLs were in the range <0.001-0.13 μg L^{-1} with MQLs being <0.001-0.43 μg L^{-1} (Table 1). In stream water, paracetamol had the greatest MQL. The sensitivity of the developed SPE-LC-MS/MS methodology is similar to those previously developed and reported in the literature for wastewaters and surface waters (Bagnall et al.,

2012; Lopez-Serna et al., 2013; Camacho-Muñoz and Kasprzyk-Hordern, 2015) (Table 2). Other than being the first enantioselective method for the determination of anthropogenic markers in septic tank effluent, the developed stereoselective LC-MS/MS method reports the greatest number of analytes in a run time ≤60 min (Table 2). Methods which do offer multi-residue enantioseparations (e.g., ≥5 analyte classes) often require run times ≥100 min (Camacho-Muñoz and Kasprzyk-Hordern, 2015; Camacho-Muñoz and Kasprzyk-Hordern, 2016). The ability to offer simultaneous determination of achiral anthropogenic markers (caffeine, paracetamol, etc) within the same methodology is a further advantage.

3.3. Anthropogenic marker stability under sample transport and storage conditions

An important consideration during development of new analytical methods is sample collection and storage. This is because errors associated with sampling can outweigh those associated with the analytical method itself (Ort et al., 2010). Grab sampling was adopted in this study to give an insight into anthropogenic marker occurrence and concentration in septic tank effluents and surrounding surface waters. However, a limitation of active sampling is the possibility for in-sample degradation or transformation of anthropogenic markers during sample transport and storage prior to processing.

Analyte stability was assessed in septic tank effluent and stream waters stored at both 18 ˚C and 4 ˚C, respectively. Results showed the studied anthropogenic markers were more stable in septic tank effluent than in stream water kept at both 18 ˚C and 4 ˚C (Figure S2; Figure 3). In septic tank effluent only aspartame fell below 75 % of its initial concentration after 48 h storage at 18 ˚C (Figure S2). On the other hand, methylparaben, carbamazepine 10,11 epoxide, triclocarban, aspartame, *S(+)*-amphetamine, *S(+)*-fluoxetine and *R(-)*-fluoxetine all fell below 75 % of their starting concentration under equivalent conditions in stream water (Figure S2). The difference in stability between the two matrices could be linked with the aerobic (stream) and anaerobic (septic tank) bacterial species present. Degradation of amphetamine in stream water was found to be stereoselective in nature due to the preferential degradation of *S(+)*-amphetamine over *R(-)*-amphetamine (Bagnall et al., 2013). An initial racemic EF of 0.5 changed to 0.1 after 48 h storage. Stereoselective change to amphetamine

has previously been observed in river water microcosms leading to the enrichment of *R(-)*-amphetamine (Bagnall et al., 2013).

Stability of anthropogenic markers was improved in both samples matrices by storing at 4 ˚C (Figure 3). These findings suggest anthropogenic marker losses during storage were biological in nature and in agreement with previous studies (Hillebrand et al., 2013; Petrie et al., 2017). In samples stored at 4 ˚C for 24 h only carbamazepine 10,11 epoxide and triclocarban degraded by ≥25 % in stream water (Figure 3B). At 4 ˚C carbamazepine 10,11 epoxide was found to be stable over 6 h. However, with practical considerations in mind a threshold of 24 h (whilst being kept at 4 ˚C) was set for the transport and processing of all samples. Under these conditions all analytes were considered stable in septic tank effluent (Figure 3A). Furthermore, no enantioselective change to chiral markers was observed in effluent or stream water stored at 4 ˚C for ≤24 h.

3.4. Application to septic tank effluents

Effluents collected from septic tanks found 10 of the studied anthropogenic chemicals were detected at least once (Figure 4). Effluent concentrations ranged from 0.07 µg L^{-1} for salbutamol-E1 to 1,600 µg L^{-1} for paracetamol. Prescription drugs showed greater spatial variation in terms of detection and concentration than observed in communal wastewater (Baker and Kasprzyk-Hordern et al., 2013; Petrie et al., 2015). This is to be expected due to the low number of people which contribute to individual septic tanks. Consequently, where detected, prescription drugs were present at comparatively greater levels than communal wastewaters.

The prescription drug found at the highest concentration was the anti-depressant citalopram. *R(-)*-citalopram and *S(+)*-citalopram were found in one of the studied effluents at 5.1 and 2.1 µg L^{-1}, respectively (Figure 4). These concentrations are >20 times greater than previously reported in communal wastewaters in the UK (Evans et al., 2015; Petrie et al., 2016b). The EF of citalopram is 0.3 and is typical for that expected in wastewater due to enantioselective metabolism in the body. The EF of other chiral drugs determined at the enantiomeric level in effluents (propranolol EF=0.40, atenolol EF=0.48 and 0.49, fluoxetine EF=0.58 and salbutamol EF=0.37 and 0.50) are typical of that previously observed in municipal wastewaters following consumption and excretion (Lopez-Serna et

al., 2013; Evans et al., 2015). To the authors knowledge chlorpheniramine has not been investigated at the enantiomeric level in wastewater before. Septic tank effluent (n=1) was found to have an enrichment of *R(-)*-chlorpheniramine (0.10 µg L^{-1} *vs.* 0.073 µg L^{-1} for *S(+)*-chlorpheniramine) and a corresponding EF of 0.4. This is contrary to pharmacokinetic studies whereby the *S(+)*-enantiomer is cleared more slowly than the *R(-)*-enantiomer resulting in an EF >0.5 in urine (chlorpheniramine is administered as a racemic mixture) (Tung Hiep et al., 1998; Yasuda et al., 2002). However, stereoselective degradation could occur within the septic tank resulting in the enrichment of the *R(-)*-enantiomer in effluent. Further investigation would be required to verify this hypothesis.

It is important to consider which anthropogenic markers can be used as indicators of rural surface water contamination by septic tanks. Three of the studied analytes were detected in >10 effluents and at high concentration. Cotinine, the metabolite of nicotine (n=12), was found in concentrations ranging from 0.14 µg L^{-1} to 21 µg L^{-1} and paracetamol (n=14) from 4.8 µg L^{-1} to 1,600 µg L^{-1} (Figure 4). However, caffeine (n=15) was determined in all samples analyzed ranging from 4.2-396 µg L^{-1}. The hydrophilic nature of caffeine (log K_{OW} -0.1) and resultant mobility in water, as well as its ubiquity in septic tank effluent make it a good indicator compound of septic tank discharge in rural surface waters. Our findings are in agreement with previous studies which have proposed caffeine as an indicator of wastewater discharge (Buerge et al., 2003; Potera, 2012), including septic tank systems (Richards et al., 2017).

3.5. Surface water quality

Surface waters were collected from two rural streams (n=11) to give insight into contamination by anthropogenic markers originating from septic tanks. In total 7 of the studied analytes were detected at least once (paracetamol, carbamazepine, carbamazepine 10,11 epoxide, cotinine, caffeine, amphetamine and atenolol). Interestingly, caffeine was detected in all stream water samples and was generally <0.5 µg L^{-1} (Table 3). Such levels are considerably lower than those observed in septic tank effluents due to further degradation (e.g., in a soak away) and dilution within the stream itself. Caffeine concentrations determined in river waters (impacted by both septic tanks and communal WWTPs) were 0.11-0.23 µg L^{-1} (Table 1). Prescription drugs detected in stream water included the

anti-epileptic carbamazepine and carbamazepine 10,11 epoxide, and the beta-blocker atenolol. These were <0.02 µg L^{-1} where quantifiable and in similar levels to that observed in the main river which is impacted by both septic tank discharges and WWTP effluent.

The most notable finding from collected stream waters was the level of anthropogenic markers found in sample 2 (Figure 1). This stream sampling site was directly after passing adjacent to several households and has low flow. Upon collection of this sample it had high turbidity and was malodorous, indicating contamination with untreated wastewater. In this sample paracetamol, cotinine and caffeine were present at 1,100 µg L^{-1}, 31 µg L^{-1} and 200 µg L^{-1}, respectively (Table 3). Such concentrations are similar to those found in septic tank effluent (Figure 4), and considerably greater than previously observed in UK surface waters. To demonstrate, the highest previously reported concentrations of paracetamol and caffeine in UK surface water is ~2 µg L^{-1} (Kasprzyk-Hordern et al., 2008; Baker and Kasprzyk-Hordern, 2013). Furthermore, *S(+)*-amphetamine and *R(-)*-amphetamine were present at 0.20 and 0.27 µg L^{-1}, respectively (Table 1). These concentrations correspond to an EF of 0.43 which is typical of that found in raw wastewater in the UK (Castrignanò et al., 2016; Castrignanò et al., 2018). Findings indicate the direct discharge of septic tank effluent (or untreated wastewater) to surface water, demonstrating the advantage of undertaking analysis at the enantiomeric level. As a limited number of samples were collected in this study to demonstrate the methods application, a more detailed investigation is now needed to better appreciate the impact of septic tanks to surrounding surface water quality.

4. Conclusion

A new multi-residue enantioselective method was successfully developed for anthropogenic markers in septic tank effluent and rural surface water for the first time. The method was adequately sensitive for 16 achiral and chiral markers within a run time of 55 min. Storage of samples at 4 ˚C was found to be sufficient for stabilising the majority of anthropogenic markers in septic tank effluent and surface water for 24 h. Application of the new methodology revealed the presence of some anthropogenic markers at high concentration in both septic tank effluents and surrounding surface waters. In rural surface water paracetamol was determined at a maximum concentration of 1,100 µg L^{-1} which is indicative of untreated wastewater discharge. Therefore, further application of the method is needed to better appreciate the environmental risk of septic tanks to surface water quality. Facilitating the simultaneous analysis of both achiral and chiral compounds at the enantiomeric level will enable a better understanding of their transport, fate and possible effects in the environment.

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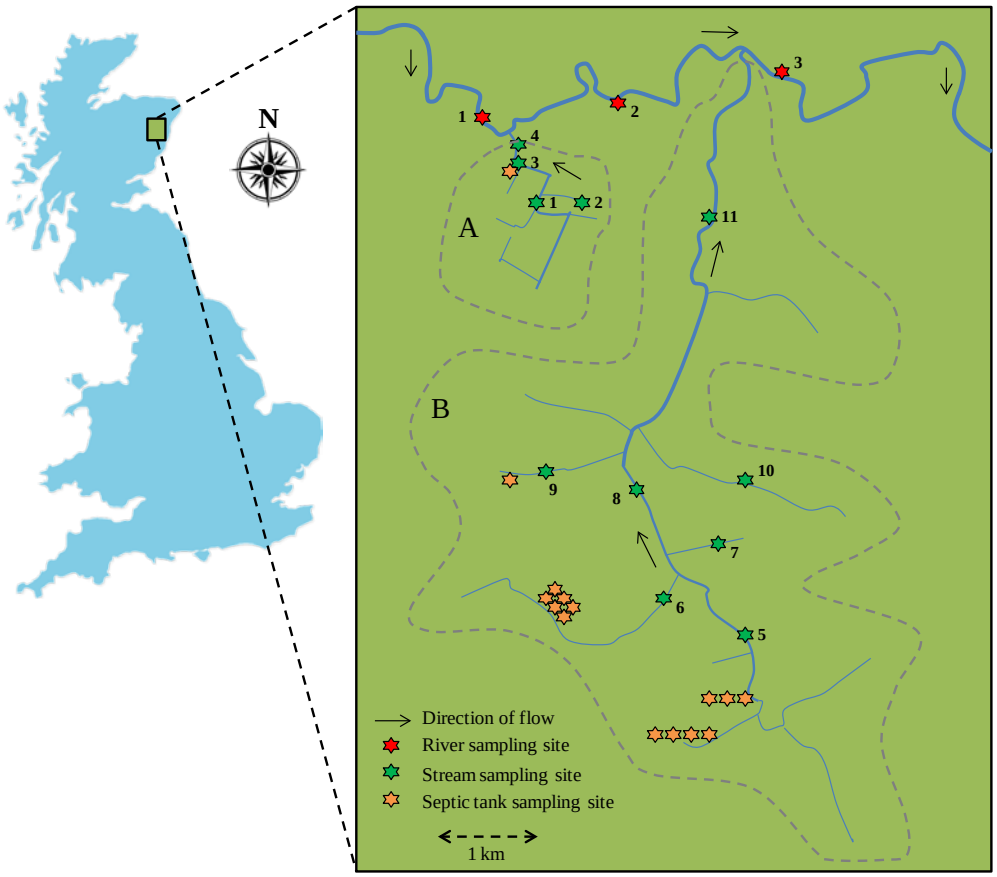

Figure 1. Area studied in North East Scotland showing septic tank and stream sampling locations within sub-catchment A and B, respectively. Sampling locations on the main river also identified.

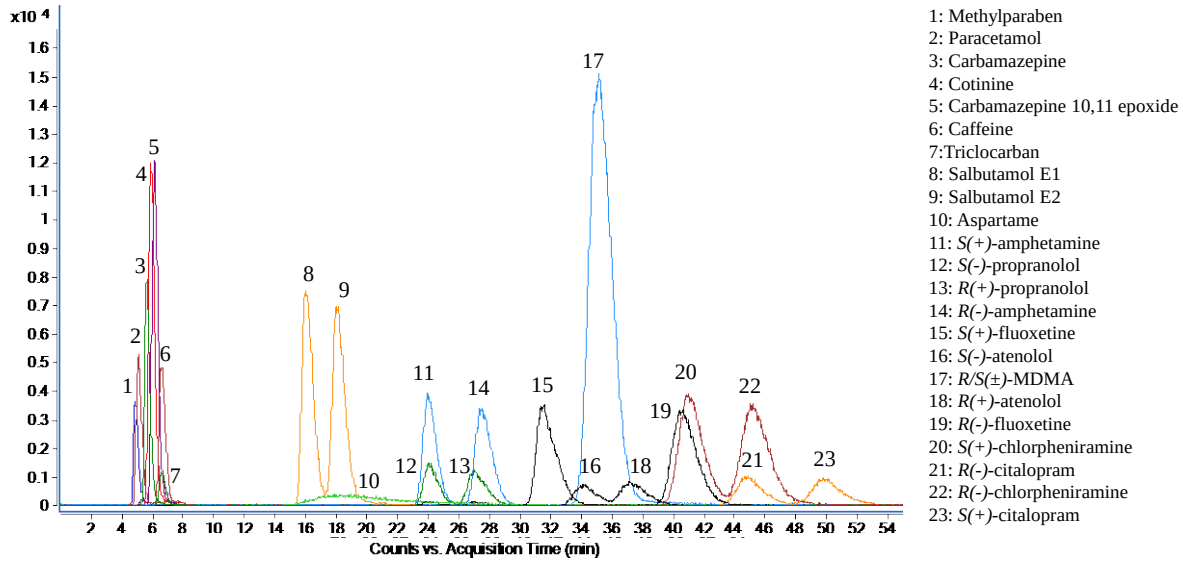

Figure 2. Multiple reaction monitoring enantioselective LC-MS/MS chromatograms of studied anthropogenic markers spiked in stream water at 0.05 µg L^{-1} (paracetamol and aspartame were spiked at 0.5 µg L^{-1}). Key: MDMA, 3,4-methylenedioxy-methamphetamine; E1, enantiomer 1; E2, enantiomer 2

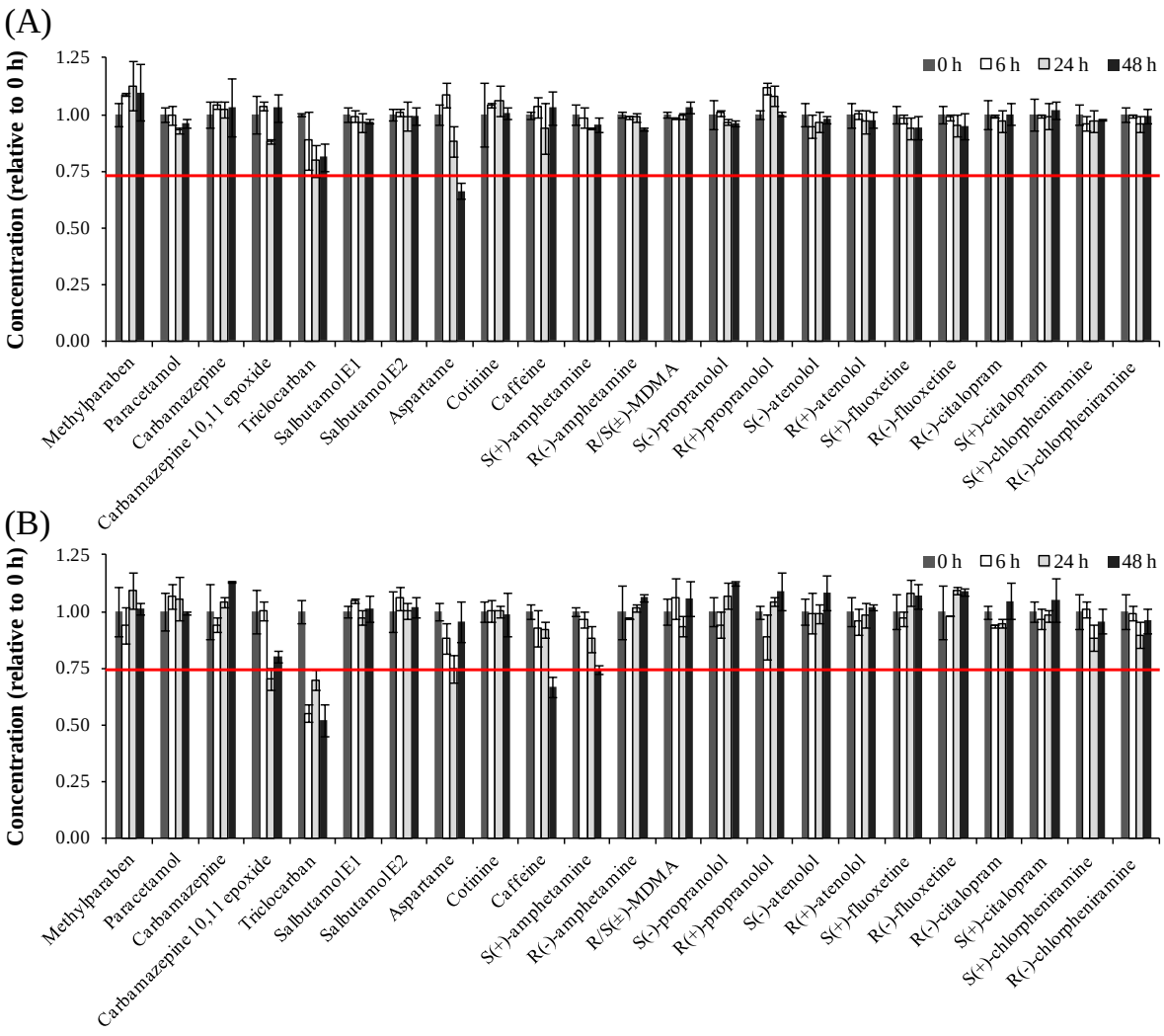

Figure 3. Stability of anthropogenic markers in septic tank effluent (A) and stream water (B) stored in polypropylene bottles stored at 4 ˚C in the dark (n=3). Key: MDMA, 3,4-methylenedioxy-methamphetamine; E1, enantiomer 1; E2, enantiomer 2

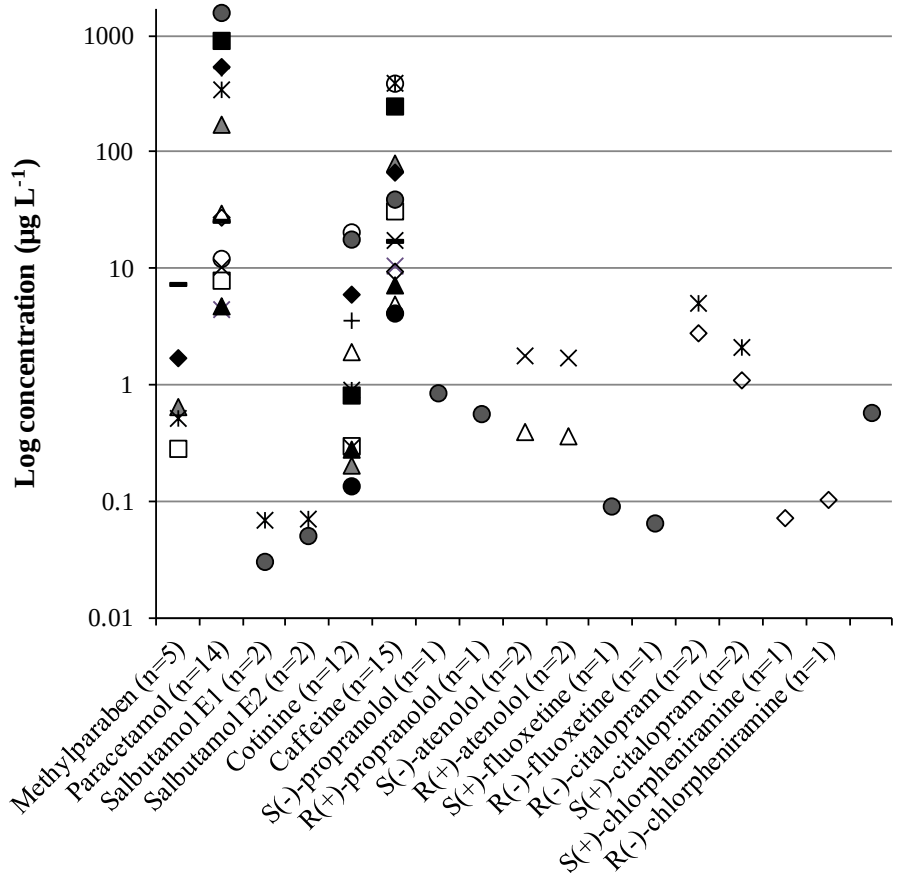

Figure 4. Anthropogenic markers determined in septic tank effluents and their concentration. Note: numbers in brackets represent the number of samples the anthropogenic marker was found in (from n=15 effluents profiled). Each effluent is represented by a different graphical marker. Key: E1, enantiomer 1; E2, enantiomer 2

Table 1. Method performance data for studied anthropogenic markers in septic tank effluent and stream water (n=3)

Anthropogenic marker class	Anthropogenic marker	Recovery from effluent (%)				Recovery from stream water (%)				Signal suppression (%)		Effluent		Stream water	
		0.5 µg L^{-1}		5 µg L^{-1}		0.05 µg L^{-1}		0.5 µg L^{-1}							
		Absolute	Corrected	Absolute	Corrected	Absolute	Corrected	Absolute	Corrected	Effluent	Stream	MDL (µg L^{-1})	MQL (µg L^{-1})	MDL (µg L^{-1})	MQL (µg L^{-1})
Preservative	Methylparaben	2±0	42±2	2±0	34±1	7±1	48±1	9±1	20±1	98±0	95±0	0.084	0.28	0.002	0.0065
Analgesic	Paracetamol	-	-	5±2	95±15	-	-	2±0	107±0	92±0	96±0	0.85	2.8	0.13	0.43
Anti-epileptic	Carbamazepine	2±0	103±7	2±0	97±2	19±1	107±1	23±4	111±4	98±0	84±1	0.075	0.25	0.0007	0.0024
	Carbamazepine 10,11 epoxide	5±1	79±5	6±0	74±5	41±2	94±2	45±2	97±2	96±0	67±1	0.0059	0.020	0.0001	0.0002
Anti-bacterial	Triclocarban	3±1	111±29	4±1	102±5	36±9	117±9	35±1	111±1	93±0	71±2	0.081	0.27	0.0008	0.0028
Beta-antagonist	Salbutamol E1	50±5	102±5	51±2	106±4	72±5	105±5	75±4	115±4	53±1	26±3	0.0016	0.0049	0.0001	0.0003
	Salbutamol E2	31±2	96±4	34±1	107±4	72±5	106±5	77±5	118±5	74±1	19±4	0.0025	0.0077	0.0001	0.0003
Sweetener	Aspartame	-	-	33±2	113±2	-	-	27±1	94±1	64±1	64±7	0.87	2.9	0.099	0.33
Stimulant and metabolite	Cotinine	5±0	91±1	2±1	100±14	16±2	117±2	23±4	105±4	82±1	76±0	0.0089	0.030	0.0002	0.0005
	Caffeine	31±9	93±4	18±8	98±6	20±4	97±5	22±7	94±7	91±3	80±9	0.0012	0.0041	0.0001	0.0005
	S(+)-amphetamine	24±2	101±2	23±3	108±2	45±4	109±4	46±3	109±3	78±2	60±9	0.0034	0.011	0.0002	0.0006
	R(-)-amphetamine	40±1	106±1	41±1	113±5	45±4	98±4	51±4	110±4	61±1	58±11	0.0020	0.0061	0.0002	0.0005
	R/S(±)-MDMA	69±2	118±3	67±1	106±2	89±6	83±36	90±3	110±3	40±2	32±3	0.0044	0.015	0.0003	0.001
Beta-blocker	*S(-)*-propranolol	28±3	101±2	27±1	98±2	94±6	101±7	87±5	108±5	74±1	23±1	0.054	0.18	0.0017	0.0056
	R(+)-propranolol	36±1	99±4	38±1	108±1	100±8	106±8	95±3	113±3	60±2	17±4	0.040	0.14	0.0015	0.0051
	S(-)-atenolol	49±3	110±10	48±2	107±2	94±8	115±8	89±2	112±2	61±1	48±3	0.0004	0.0010	0.0001	0.0003
	R(+)-atenolol	94±2	110±4	99±2	107±4	116±8	101±8	110±4	110±4	44±2	26±3	0.0002	0.0005	0.0001	0.0003
Anti-depressant	*S(+)*-fluoxetine	29±1	104±3	31±1	107±3	34±3	107±3	37±2	114±2	66±0	70±4	0.0050	0.017	0.0042	0.14
	R(-)-fluoxetine	53±3	104±4	59±1	106±3	66±4	104±4	61±3	109±3	40±1	38±6	0.0027	0.0090	0.0002	0.0008
	R(-)-citalopram	66±2	110±3	68±1	111±4	93±2	101±2	92±4	102±4	30±0	18±3	0.023	0.075	0.0016	0.0054
	S(+)-citalopram	70±3	116±4	76±1	111±7	96±5	92±6	96±2	106±2	20±2	16±0	0.021	0.069	0.0016	0.0052
Anti-histamine	*S(+)*-chlorpheniramine	78±1	104±1	84±1	104±1	102±5	108±5	102±4	110±4	20±4	7±7	0.019	0.062	0.0015	0.0049
	R(-)-chlorpheniramine	85±2	104±2	90±1	111±3	99±7	99±7	107±5	113±6	20±4	7±5	0.017	0.057	0.0015	0.0049

Key: MDL, method detection limit; MQL, method quantitation limit; MDMA, 3,4-methylenedioxy-methamphetamine; E1, enantiomer 1; E2, enantiomer 2

Table 2. Enantioselective LC-MS/MS methods validated for the determination of anthropogenic markers in wastewaters and surface waters

Anthropogenic markers	Sample type + preparation	Chromatographic column	Mobile phase conditions	Run time (min)	MS detector	Enantiomer R_S	Method recovery (%)	MDL (µg L^{-1})	Reference
Aminorex, carboxyibuprofen, cephalexin, chloramphenicol, dechloroethylifosfamide, O-desmethylnaproxen, 10,11-dihydro-10-hydroxy carbamazepine, dihydroketoprofen, florfenicol, griseofuvlin, 2-hydroxyibuprofen, ibuprofen, ifosfamide, indoprofen, ketoprofen, naproxen, phenylpropionic acid, praziquantel & tetramisole	River water (200 mL), wastewater effluent (100 mL) filtered (0.7 µm) and Oasis HLB-MAX SPE. Reconstituted in 0.5 mL mobile phase	Chirobiotic T® 250 x 4.6 mm, I.D. 5 µm @ 25 °C	10 mM ammonium acetate in water (pH 4.2): methanol (70:30, *v/v*) @ 0.08 mL min^{-1}	150	QqQ	0.4-0.9	8-127	0.0001-1.3	Camacho-Muñoz and Kasprzyk-Hordern, 2017
Omeprazole*, lansoprazole*, pantoprazole* & rabeprazole*	Wastewater/river water (100 mL) adjusted to pH 10 and Cleanert PEP-2 SPE & DLLME	Chiralpak IC® 250 x 4.6 mm, I.D. 5 µm	Acetontrile:5 mM ammonium acetate in water (40:60, *v/v*) @ 0.6 mL min^{-1}	30	QqQ	>1.5	90-107	0.0007-0.0023	Zhao et al., 2016
Aminorex*, carboxyibuprofen, cephalexin, chloramphenicol*, dechloroethylifosfamide, 10,11-dihydro-10-hydroxy carbamazepine, dihydroketoprofen*, fexofenadine*, 2-hydroxyibuprofen, ibuprofen*, ifosfamide*, indoprofen, ketoprofen, mandelic acid, naproxen*, phenylpropionic acid, praziquantel & tetramisole*	River water (500 mL), wastewater effluent (250 mL) filtered (0.7 µm) and Oasis HLB-MAX SPE. Reconstituted in 0.5 mL mobile phase	Chiral AGP 100 x 2 mm, I.D. 5 µm @ 25 °C	10 mM ammonium acetate in water with 1 % acetonitrile (pH 6.7)	100	QqQ	≥0.7	2-158	0.0001-0.34	Camacho-Muñoz and Kasprzyk-Hordern, 2015
Flumequine, albuterol*, ketoprofen, pindolol*, propranolol*, atenolol*, metoprolol*, clenbuterol*, sotalol*, timolol*, naproxen & fluoxetine*	River water (500 mL), wastewater effluent (100 mL) filtered (0.7 µm) and Oasis HLB SPE. Reconstituted in 0.5 mL mobile phase	Chirobiotic V® 250 x 4.6 mm, I.D. 5 µm @ 25 °C	4 mM ammonium acetate + 0.005 % formic acid in methanol @ 0.1 mL min^{-1}	65	QqQ	≥0.4-1.1	56-116	0.0001-0.011	Lopez-Serna et al., 2013
Amphetamine*, methamphetamine*, MDMA*, propranolol*, atenolol*, metoprolol*, venlafaxine* & fluoxetine*	River water (250 mL), effluent (100 mL) filtered (0.7 µm) and Oasis HLB SPE. Reconstituted in 0.5 mL mobile phase	Chirobiotic V® 250 x 4.6 mm, I.D. 5 µm @ 25 °C	4 mM ammonium acetate + 0.005 % formic acid in methanol @ 0.1 mL min^{-1}	40	QTOF-MS	0.9-4.7	61-126	0.0002-0.023	Bagnall et al., 2012
Aspartame, caffeine, carbamazepine, carbamazepine 10,11 epoxide, cotinine, methylparaben, paracetamol, triclocarban, amphetamine*, atenolol*, chlorpheniramine*, citalopram*, fluoxetine*, MDMA, propranolol* & salbutamol*	River water (250 mL), septic tank effluent (25 mL) filtered (0.7 µm) and Oasis HLB SPE. Reconstituted in 0.25 mL mobile phase	Chirobiotic V2® 250 x 2.1 mm, I.D. 5 µm @ 15 °C	1 mM ammonium acetate + 0.01 % acetic acid in methanol @ 0.17 mL min^{-1}	60	QqQ	1-2.3	20-118	0.0001-0.87	This study

Key: MS/MS, tandem mass spectrometry; MDL, method detection limit; QqQ, triple quadrupole; SPE, solid phase extraction; NH_4OAc, ammonium acetate; MeOH, methanol; ACN, acetonitrile; HCOOH, formic acid; CH_3COOH, acetic acid; QTOF, quadrupole time of flight; MDMA, 3,4-methylenedioxy-methamphetamine; *, highlights those separated at the enantiomeric level with $R_S \geq 1$

Table 3. Concentration of anthropogenic markers detected in studied surface water samples (µg L^{-1})

Anthropogenic marker	Stream water sample											River water sample		
	Sub-catchment A				Sub-catchment B									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**1**	**2**	**3**
Paracetamol	<MQL	1,100	<MQL	<MQL	1.6	<MQL	<MQL	<MQL	1.0	<MQL	<MQL	<MQL	<MQL	<MQL
Carbamazepine	-	-	0.0037	-	-	-	0.011	-	-	-	0.0091	0.015	0.015	0.012
Carbamazepine 10,11 epoxide	-	-	0.0056	-	-	-	<MQL	-	-	-	<MQL	0.018	0.013	0.011
Cotinine	-	31	0.0063	<MQL	0.013	0.011	0.0015	0.0025	0.011	0.00070	0.012	0.0042	0.0025	0.0011
Caffeine	0.036	200	0.16	0.038	0.49	0.17	0.29	0.17	0.37	0.045	0.42	0.19	0.23	0.11
S(+)-amphetamine	-	0.20	-	-	-	-	-	-	-	-	-	-	-	-
R(-)-amphetamine	-	0.27	-	-	-	-	-	-	-	-	-	-	-	-
S(-)-atenolol	-	-	-	-	0.015	-	-	0.0054	-	-	0.020	0.0056	0.0056	0.0039
R(+)-atenolol	-	-	-	-	0.015	-	-	0.0043	-	-	0.019	0.0045	0.0049	0.0034

Key: -, below method detection limit; <MQL, below method quantitation limit

Note: Sample locations correspond to those outlined in the catchment map in Figure 1. All other anthropogenic markers were not detected in any of the collected surface water samples

Supplementary information

Enantioselective LC-MS/MS for anthropogenic markers of septic tank discharge

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The supplementary information contains two figures and three tables which contains information on the impact of sample pre-concentration factor to anthoprogenic marker response, the stability of anthropogenic markers in septic tank effluent and stream water stored at 18 °C, MS/MS parameters and instrumental performance data.

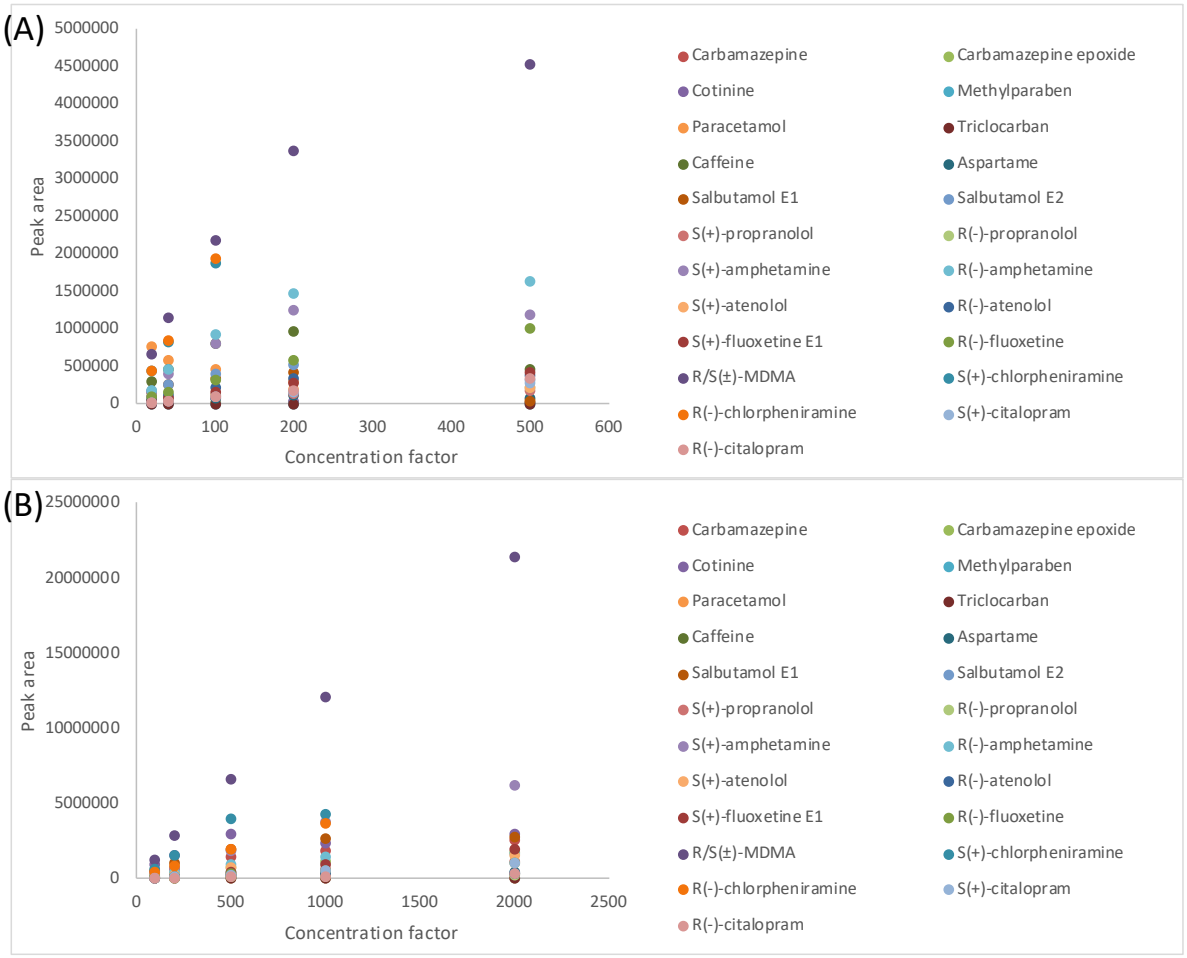

Figure S1. Impact of SPE concentration factor on analyte response for septic tank effluent (A) and stream water (B). Key: MDMA, 3,4-methylenedioxy-methamphetamine; E1, enantiomer 1; E2, enantiomer 2

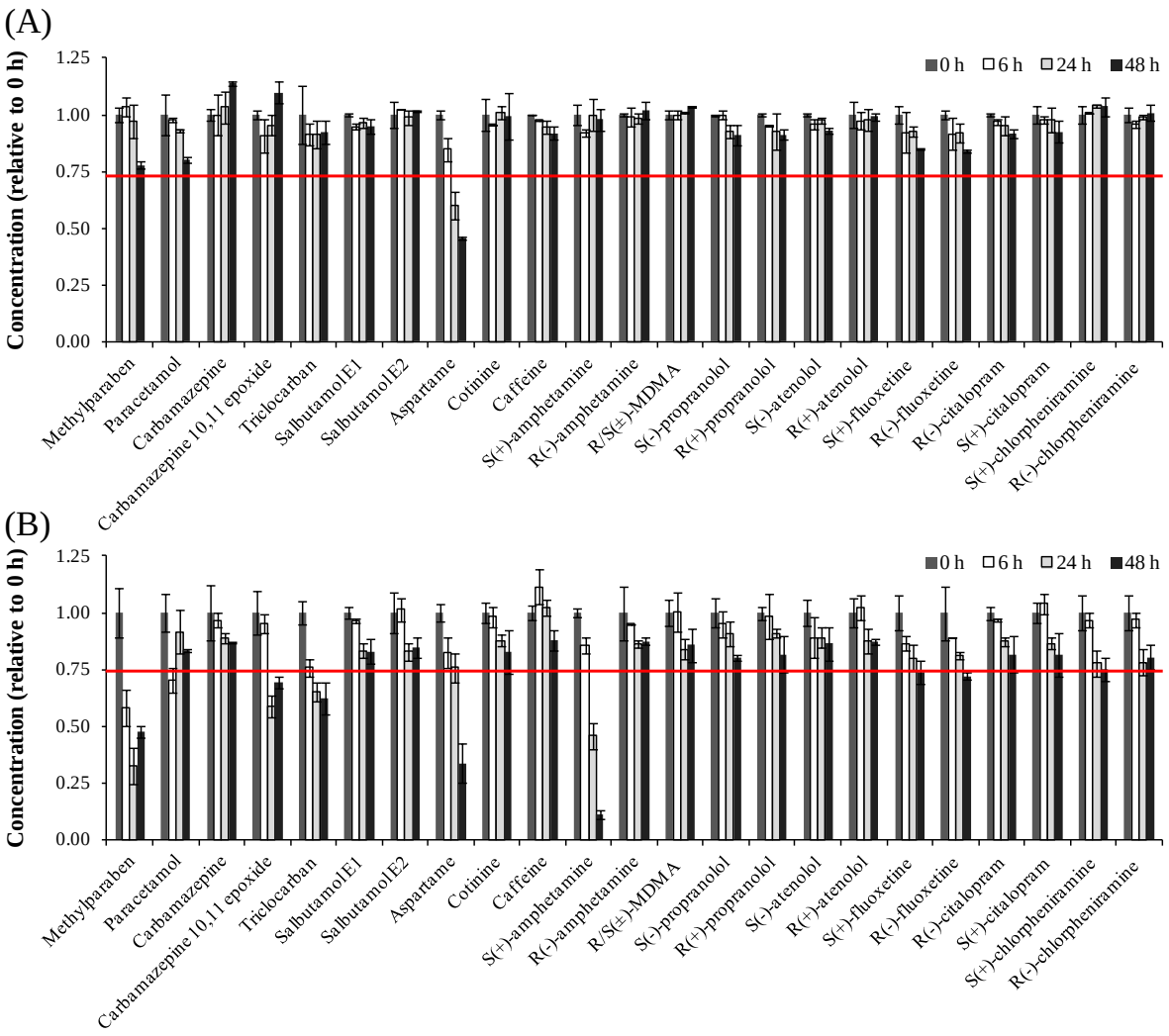

Figure S2. Stability of anthropogenic markers in septic tank effluent (A) and stream water (B) stored in polypropylene bottles stored at 18 ˚C in the dark. Key: MDMA, 3,4-methylenedioxy-methamphetamine; E1, enantiomer 1; E2, enantiomer 2

Table S1. MS/MS method detail for studied anthropogenic markers

Class of anthropogenic marker	Anthropogenic marker	Fragmentor (V)	MRM 1 (quantifier)	Collision energy (eV)	MRM 2 (qualifier)	Collision energy (eV)	Corresponding internal standard
Preservative	Methylparaben	90	150.9>92.0	20	150.9>136.0	10	Caffeine-13C3
Analgesic	Paracetamol	100	151.9>110.0	10	151.9>65.1	30	Paracetamol-d4
Anti-epileptic	Carbamazepine	130	236.8>178.9	40	236.8>193.9	20	Carbamazepine-d10
	Carbamazepine 10,11 epoxide	90	252.8>179.9	30	252.8>210.0	10	Carbamazepine 10,11 epoxide-d10
Anti-bacterial	Triclocarban	110	312.5>159.7	10	312.5>125.6	20	Triclocarban-d3
Beta-antagonist	Salbutamol	90	239.9>147.9	10	239.9>165.9	10	Salbutamol-d3
Sweetener	Aspartame	90	295.0>119.9	20	295.0>180.0	30	*S(+)*-fluoxetine-d6
Stimulant and metabolite	Cotinine	90	176.9>80.0	20	176.9>98.0	20	Cotinine-d3
	Caffeine	90	194.9>110.0	20	194.9>138.0	18	Caffeine-13C3
	R/S(±)-amphetamine	70	135.8>90.9	20	135.8>65.0	40	*R/S(±)*-amphetamine-d11
	R/S(±)-MDMA	90	193.9>162.8	10	193.9>104.8	30	*R/S(±)*-MDMA-d5
Beta-blocker	*R/S(±)*-propranolol	110	259.9>115.9	30	259.9>182.9	20	*R/S(±)*-propranolol-d7
	R/S(±)-atenolol	90	267.0>145.0	30	267.0>190.0	20	*R/S(±)*-atenolol-d7
Anti-depressant	*R/S(±)*-fluoxetine	90	309.8>44.0	10	309.8>147.7	5	*R/S(±)*-fluoxetine-d6
	R/S(±)-citalopram	130	325.0>108.9	30	325.0>262.0	20	*R/S(±)*-citalopram-d6
Anti-histamine	*R/S(±)*-chlorpheniramine	90	274.9>229.9	10	274.9>166.8	40	*R/S(±)*-chlorpheniramine-d6
Labelled surrogates	Caffeine-13C3	90	198.0>139.9	20	-	-	-
	Paracetamol-d4	90	155.9>114.0	20	-	-	-
	Carbamazepine-d10	130	246.9>204.1	20	-	-	-
	Carbamazepine 10,11 epoxide-d10	90	263.0>189.9	30	-	-	-
	Triclocarban-d3	110	318.9>161.9	10	-	-	-
	Salbutamol-d3	90	243.0>150.9	10	-	-	-
	R/S(±)-amphetamine-d11	70	147.0>98.0	20	-	-	-
	R/S(±)-MDMA-d5	90	199.0>164.9	10	-	-	-
	R/S(±)-propranolol-d7	110	267.0>188.8	15	-	-	-
	Cotinine-d3	90	180.0>80.0	30	-	-	-
	R/S(±)-fluoxetine-d6	90	316.0>154.0	2	-	-	-
	R/S(±)-atenolol-d7	100	274.1>145.0	30	-	-	-
	R/S(±)-citalopram-d6	130	331.0>109.0	30	-	-	-
	R/S(±)-chlorpheniramine-d6	100	281.0>229.9	10	-	-	-

Key: MRM, multiple reaction monitoring; MDMA, 3,4-methylenedioxy-methamphetamine; E1, enantiomer 1; E2, enantiomer 2

Table S2. Instrument performance information for studied anthropogenic markers

Class of anthropogenic marker	Anthropogenic marker	*Rt* (min)	Linearity		$IDL_{S/N}$ (µg L^{-1})	$IQL_{S/N}$ (µg L^{-1})
			Range (µg L^{-1})	r^2		
Preservative	Methylparaben	4.78±0.02	0.50-500	0.999	0.15	0.50
Analgesic	Paracetamol	4.98±0.03	10-1,000	0.999	3.0	10
Anti-epileptic	Carbamazepine	5.47±0.01	0.50-2,000	0.999	0.15	0.50
	Carbamazepine 10,11 epoxide	6.06±0.03	0.10-1,000	0.999	0.030	0.10
Anti-bacterial	Triclocarban	6.48±0.03	1.0-500	0.999	0.30	1.00
Beta-antagonist	Salbutamol E1	16.10±0.17	0.25-2,500	0.999	0.080	0.25
	Salbutamol E2	18.14±0.21	0.25-2,500	0.999	0.080	0.25
Sweetener	Aspartame	20.60±0.78	100-1,000	0.999	30	100
Stimulant and metabolites	Cotinine	5.85±0.03	0.10-1,000	0.999	0.030	0.10
	Caffeine	6.46±0.03	0.10-1,000	0.999	0.030	0.10
	S(+)-amphetamine	24.33±0.27	0.25-2,500	0.999	0.080	0.25
	R(-)-amphetamine	28.04±0.33	0.25-2,500	1.000	0.080	0.25
	R/S(±)-MDMA	35.77±0.45	1.0-1,000	0.999	0.30	1.0
Beta-blocker	*S(-)*-propranolol	24.42±0.32	5.0-1,000	0.999	1.5	5.0
	R(+)-propranolol	27.50±0.35	5.0-1,000	0.999	1.5	5.0
	S(-)-atenolol	34.75±0.44	0.050-1,000	0.999	0.020	0.050
	R(+)-atenolol	37.91±0.46	0.050-1,000	0.999	0.020	0.050
Anti-depressant	*S(+)*-fluoxetine	32.03±0.40	0.50-2,500	0.999	0.15	0.50
	R(-)-fluoxetine	41.54±0.60	0.50-2,500	0.999	0.15	0.50
	R(-)-citalopram	45.60±0.77	5.0-1,000	0.999	1.5	5.0
	S(+)-citalopram	50.93±0.93	5.0-1,000	0.999	1.5	5.0
Anti-histamine	*S(+)*-chlorpheniramine	42.19±0.76	5.0-1,000	0.999	1.5	5.0
	R(-)-chlorpheniramine	46.53±0.88	5.0-1,000	0.999	1.5	5.0

Key: *Rt*, retention time; IDL, instrument detection limit; IQL, instrument quantitation limit; MDMA, 3,4-methylenedioxy-methamphetamine; E1, enantiomer 1; E2, enantiomer 2

Table S3. Inter-day and intra-day precision and accuracy of enantioselective LC-MS/MS method

Class of anthropogenic marker	Anthropogenic marker	Precision (%, expressed as RSD)						Accuracy (%)					
		Intra-day			Inter-day			Intra-day			Inter-day		
		Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High
Preservative	Methylparaben	3.6	0.7	0.5	4.6	0.1	0.3	96.9	99.6	101.1	98.7	99.2	100.1
Analgesic	Paracetamol	3.4	1.8	1.2	3.5	0.8	0.7	95.0	99.7	101.4	94.8	101.5	102.6
Anti-epileptic	Carbamazepine	6.9	4.7	3.1	8.1	1.1	1.7	112.9	110.0	99.9	110.5	106.6	102.4
	Carbamazepine 10,11 epoxide	0.6	2.4	1.8	1.2	0.2	0.6	92.4	99.7	97.9	93.2	99.5	97.4
Anti-bacterial	Triclocarban	3.8	3.2	4.0	0.9	3.2	4.0	92.9	96.6	92.7	90.8	96.6	92.7
Beta-antagonist	Salbutamol E1	0.8	2.9	1.8	0.3	1.0	0.7	96.7	95.2	104.2	95.6	96.9	102.7
	Salbutamol E2	1.5	0.4	4.2	1.6	3.0	2.9	104.4	106.1	94.9	106.0	109.7	93.4
Sweetener	Aspartame	3.2	1.6	0.6	4.9	3.2	1.5	101.6	89.4	97.8	103.7	91.9	98.7
Stimulant and metabolites	Cotinine	0.6	2.4	1.8	1.2	0.2	0.6	92.4	99.7	97.9	93.2	99.5	97.4
	Caffeine	3.2	1.3	1.1	1.3	0.6	0.1	89.0	100.8	99.5	87.9	98.7	100.1
	S(+)-amphetamine	2.8	3.3	0.2	0.8	0.3	0.2	93.3	98.8	101.4	93.4	100.7	99.4
	R(-)-amphetamine	2.2	1.8	0.4	1.1	3.2	2.3	93.0	98.9	97.6	94.6	101.0	99.0
	R/S(±)-MDMA	1.5	5.3	0.1	0.4	2.3	1.8	94.1	98.8	96.0	95.0	98.7	97.1
Beta-blocker	*S(-)*-propranolol	3.2	0.8	0.4	1.1	0.7	0.2	98.2	95.4	99.2	98.7	95.6	99.6
	R(+)-propranolol	4.4	2.2	1.4	1.1	0.5	1.4	91.3	90.6	98.3	85.6	93.4	98.3
	S(-)-atenolol	1.6	1.1	2.0	1.4	1.4	2.3	95.8	102.3	97.6	94.8	99.7	102.0
	R(+)-atenolol	3.3	1.4	1.7	1.8	2.4	1.1	93.8	98.3	106.0	92.8	103.3	103.8
Anti-depressant	*S(+)*-fluoxetine	0.9	2.0	2.1	0.9	1.8	2.0	91.5	100.7	99.0	91.5	100.6	99.7
	R(-)-fluoxetine	2.3	1.8	1.2	2.8	3.1	1.3	90.4	99.6	101.0	92.4	102.3	102.4
	R(-)-citalopram	1.3	2.0	1.1	1.1	1.5	1.1	105.9	96.1	97.2	104.6	94.3	97.2
	S(+)-citalopram	1.9	2.0	0.5	1.5	1.3	0.1	102.7	109.6	107.3	100.9	109.0	106.7
Anti-histamine	*S(+)*-chlorpheniramine	0.9	1.3	0.3	0.9	0.6	0.3	99.4	107.6	110.0	98.9	106.8	110.0
	R(-)-chlorpheniramine	2.0	1.0	0.8	0.8	1.7	0.9	94.2	98.4	97.7	95.7	99.7	98.3

Key: MDMA, 3,4-methylenedioxy-methamphetamine; E1, enantiomer 1; E2, enantiomer 2; RSD, relative standard deviation; Low, mid and high concentration levels were 0.010, 0.10 and 0.50 ng mL^{-1}, respectively. For aspartame the concentration levels were 0.010, 0.10 and 0.50 ng mL^{-1}