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Estimation of community-wide drugs use via stereoselective profiling of sewage

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Abstract

This paper explores possibilities of applying enantiomeric profiling to solving problems related to estimation of drugs usage in communities via the *sewage epidemiology* approach: for the identification of whether drug residue results from consumption of illicit drug or metabolism of other drugs, verification of potency of used drugs and monitoring of changing patterns of drugs abuse. Due to the very complex nature of wastewater used in sewage epidemiology, which comes from the whole community rather than one individual, verification of the above is challenging but vital in accurate estimations of drugs abuse as well as providing comprehensive information regarding drug abuse trends. The results of this study indicated that amphetamine in raw wastewater was enriched with *R*(-)-enantiomer due to its abuse as racemate. Methamphetamine was found to be racemic or to be enriched with *S*(+)-enantiomer. MDMA was enriched with *R*(-)-MDMA, which was to be expected as MDMA is abused as racemate. MDA was enriched with *S*(+)-enantiomer, which suggests that its presence might be associated with MDMA abuse and not intentional MDA use. Out of the four possible isomers of ephedrine only natural *1R,2S*(-)-ephedrine and *1S,2S*(+)-pseudoephedrine were detected in raw wastewater and their diastereomeric fractions were found to be season dependent with higher contribution from *1S,2S*(+)-pseudoephedrine over winter months and an enrichment with *1R,2S*(-)-ephedrine during the spring and summer months. These findings were accompanied by a decrease of cumulative concentration of ephedrines throughout the sampling campaign between February and August. This is a very important finding indicating that non-enantioselective measurement of ephedrine concentrations cannot be a reliable indicator of actual potency of ephedrines used.

Keywords: sewage, drug epidemiology, chiral, drugs of abuse, enantiomeric profiling, wastewater analysis, environment, amphetamines

1. Introduction

'*Sewage epidemiology*' is a new and very promising approach for the estimation of drugs consumption in communities via the analysis of sewage. It was first proposed by Daughton in 2001 (Daughton 2001), implemented by Zuccato et al. in 2004 (Zuccato, Chiabrando et al. 2005) and followed by others (Bones, Thomas et al. 2007; Huerta-Fontela, Galceran et al. 2008; Banta-Green, Field et al. 2009; Kasprzyk-Hordern, Dinsdale et al. 2009; Mari, Politi et al. 2009; van Nuijs, Pecceu et al. 2009; Karolak, Nefau et al. 2010; Metcalfe, Tindale et al. 2010; Postigo, de Alda et al. 2010; Terzic, Senta et al. 2010; Harman, Reid et al. 2011). Those wanting to find out more about this new concept are referred to existing reviews (Postigo, Lopez de Ada et al. 2008; Zuccato, Chiabrando et al. 2008; van Nuijs, Castiglioni et al. 2010). Significant advances in the development

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of this new tool were observed in recent years but there are still many uncertainties mainly connected with the not entirely understood fate of drugs and their metabolites in sewers, and the varying and not well understood excretion profiles of drugs, which might be altered by disease, drug interactions, ethnic differences, sex, age and lifestyle. Other sewage epidemiology biases are discussed elsewhere (van Nuijs, Castiglioni et al. 2010; Lai, Ort et al. 2011). The above uncertainties need to be resolved before this tool is implemented as a routine population screening technique for drugs use. One aspect of drugs properties, namely chirality, has not been previously taken into account in sewage epidemiology, despite its critical importance in drugs' abuse potency and toxicity, as well as their disposition (distribution, metabolism and excretion) in the body.

Most illicit drugs are chiral compounds. Among them are plant-derived substances (e.g. cannabis, cocaine and heroin) and synthetic drugs (e.g. amphetamine, methamphetamine and related designer drugs). A chiral molecule usually has at least one chiral centre (e.g. asymmetric carbon) as a result of which it shows optical activity. It exists in the form of two enantiomers, being the non-superimposable mirror images of each other. Enantiomers of the same drug have similar physico-chemical properties but differ in their biological properties such as distribution, metabolism and excretion, as these processes (due to stereospecific interactions of enantiomers with biological systems) usually favour one enantiomer over the other. Additionally, due to different pharmacological activity, chiral drugs can differ in toxicity. *R,R*(+)-LSD is for example over 20 times more psychoactive than (-)-LSD. Cocaine, similarly to heroin, naturally occurs in the form of *1R,2R,3S,5S*(-)-cocaine. (+)-Cocaine (the unnatural enantiomer) is inactive. Both metabolism and toxicity of (+)- and (-)-cocaine were found to be stereoselective. In cannabinoids, the natural *d*-1-THC and *d*-6-THC have a (*3R,4R*) configuration and a negative rotation. Synthetic (+)-isomers are much less active. For instance (+)-*d*-1-THC is ca. 13 to 230 times less active than the (-)-isomer in cannabimimetic activity (Kasprzyk-Hordern 2010).

The phenomenon of chirality is often utilised by forensic scientists in for example amphetamine or methamphetamine abuse cases, where distinction between legal and illicit usage has to be made. It can also help with identifying synthetic routes of clandestine manufacture of these drugs. It might be of vital importance in sewage epidemiology in:

- Distinction between legal and illicit use of drugs.
- Verification of the method of synthesis of illicit drugs.
- Identification of whether drug residue results from consumption of illicit drug or metabolism of other (illicit) drug.
- Verification of route of administration.
- Verification of potency of abused drugs.
- Monitoring of changing patterns of drugs abuse.

This paper aims to explore the possibilities of applying enantiomeric profiling to solving problems related to estimation of drugs usage in communities via the *sewage epidemiology* approach. To the authors' knowledge this is the first attempt to undertake such studies in *sewage epidemiology* and therefore the aim of this paper is to draw attention to the problem rather than to give solutions.

2. Experimental

2.1. Chemicals and materials

All reference standards (*R*(-)-amphetamine, *S*(+)-amphetamine, *R*/*S*(±)-amphetamine, *R*(-)-methamphetamine, *S*(+)-methamphetamine, *1R,2S*(-)-ephedrine, *1S,2S*(+)-pseudoephedrine, *R*/*S*(±)-MDA, *R*/*S*(±)-MDMA) and internal standards (IS): (*R*/*S*(±)-amphetamine-d11, *R*/*S*(±)-methamphetamine-d14, *R*/*S*(±)-MDMA-d5, *R*/*S*(±)-MDA-d5) (Table 1) were purchased from LGC

Standards (Teddington, UK) and Sigma-Aldrich (Gillingham, UK). All solvents used were of LC or LC/MS quality. All internal standards were added to the samples before extraction and were also used for the quantification of the analytes.

2.2. Sampling

Grab wastewater samples (2 per each sampling point) were collected from seven WWTPs in England (Tab. 2) over the period of 8 months (January 2010 – August 2010) during 5 sampling campaigns. Samples were collected in 2.5 L silanized amber bottles with Teflon faced phenolic caps. The samples were primarily filtered through GF/D 2.7 μm glass fibre filter (Whatman, UK) and subsequently through 0.7 μm glass fibre filter GF/F (Whatman, UK).

2.3. Sample preparation and analysis

2.3.1. Enantiomeric profiling of chiral drugs with SPE-Chiral LC-MS/MS

Chiral drugs were extracted from wastewater using SPE Gilson ASPEC XL4 (Anachem, UK) and Oasis HLB adsorbents (Waters, UK). A volume of 100 mL of filtered wastewater samples (pH, 7.5 adjusted with NaOH) spiked with 100 ng of internal standards was passed through the cartridge at a rate of 6 mL/min. Analytes were extracted from HLB cartridge with 4 mL of MeOH at a rate of 1 mL/min. The extracts were evaporated to dryness with TurboVap evaporator (Caliper, UK, 40^{o}C, N_2, 5-15 psi) and finally reconstituted in 0.5 mL of mobile phase. All samples were filtered through 0.2 μm PTFE filters (Whatman, Puradisc, 13 mm) and transferred to maximum recovery deactivated vials with PTFE septa (Waters, UK).

Waters ACQUITY UPLC^{TM} system (Waters, Manchester, UK) consisting of ACQUITY UPLC^{TM} binary solvent manager and ACQUITY UPLC^{TM} sample manager was used for the separation of analytes. Chiral-CBH column, 100x2mm, 5μm (Chromtech, Congleton, UK) and Chiral-CBH 10x2.0mm guard column (Chromtech, Congleton, UK) were used for the separation of enantiomers of chiral drugs. The separation of chiral drugs was undertaken under isocratic conditions with the usage of mobile phase (pH, 5.0) composed of 90% H_2O, 10% 2-propanol and 1 mM ammonium acetate. 20 μL of the sample was injected into the system. The column was kept at 25^{o}C and the temperature in the sample manager was kept at 4^{o}C. The flow rate of mobile phase was 0.075 mL/min, which allowed for the introduction of mobile phase from LC into MS without splitting.

A TQD (triple quadrupole) mass spectrometer (Waters, Manchester, UK), equipped with an electrospray ionisation source, was used for drugs of abuse quantification. The analyses were performed in positive mode with a capillary voltage of 3 kV, a source temperature of 150^{o}C and a desolvation temperature of 200^{o}C. A cone gas flow of 50 L/h and desolvation gas flow of 450 L/h were used. Nitrogen, used as a nebulising and desolvation gas, was provided by a high purity nitrogen generator (Peak Scientific Instruments Ltd, UK). Argon (99.999%) was used as a collision gas. MassLynx 4.1 (Waters, UK) software was used to collect and analyse the obtained data. Mass spectrometry analyses were performed in the multiple reaction monitoring (MRM) mode, measuring the fragmentation of the protonated pseudo-molecular ions of each chiral drug (Tab. 3). A dwell time of 50 ms per ion pair was used to maintain high sensitivity of the analysis and required a number of data points across the chromatographic peak. All instrumental and methods validation parameters such as: linearity and range, accuracy, precision, detection and quantification limits and calibration curve were determined. A detailed discussion of this method is presented elsewhere (Kasprzyk-Hordern, Kondakal et al. 2010). Validation parameters for this method can be found in Tab. 4.

The relative concentration of enantiomers of chiral drugs was expressed as the enantiomeric fraction (*EF*) and was calculated with the following equation:

$$EF = \frac{R(-)}{R(-) + S(+)}$$

where *R*(-) and *R*(+) are peak areas for *R*(-) and *S*(+) enantiomers of a chiral drug. *EF* equals 1 or 0 in the case of single enantiomer form and 0.5 in the case of racemate. Enantiomers of amphetamine, ephedrine and methamphetamine were confirmed in this research through the usage of enantiomerically pure standards. Identification of elution order of MDMA and MDA was based on data published by others (Fornstedt, Hesselgren et al. 1997; Buechler, Schwab et al. 2003).

Out of two enantiomeric pairs of (pseudo)ephedrine only two diastereomers: 1*R*,2*S*(-)-ephedrine and 1*S*,2*S*(+)-pseudoephedrine were frequently quantified in wastewater. For these two compounds diastereomeric fractions (*DFs*) were calculated:

$$DF = \frac{1R,2S(-)\text{-}ephedrine}{1R,2S(-)\text{-}ephedrine + 1S,2S(+)\text{-}pseudoephedrine}$$

2.3.2. Quantification of chiral drugs with SPE-UPLC-MS/MS

Solid phase extraction of samples was carried out as described above. Oasis MCX cartridge was used for the extraction of all analytes. 500 mL of acidified river water and 100 mL of wastewater were spiked with 50 ng of each surrogate/internal standard and then passed through the MCX cartridge at a rate of 6 mL min^{-1}. Cartridges were washed with 0.6%HCOOH/MeOH (2 mL, pH 2) at a flow rate of 3 mL min^{-1} followed by elution with 7%NH_4OH/MeOH (3 mL) at a flow rate of 1 mL min^{-1} into silanised vials. Extracts were evaporated to dryness (40 °C, N_2, 2-10 psi) and reconstituted with 0.3%CH_3COOH/5%MeOH/H_2O (500 µL). All samples were filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13 mm) before being transferred to maximum recovery deactivated vials with PTFE septa (Waters, UK).

Analyses were carried out with the usage of Waters ACQUITY UPLC™ system (Waters, UK) and ACQUITY UPLC BEH C18, 150x1mm, 1.7µm columns (Waters, UK). The UPLC method employed mobile phase A (pH 2.9): 79.7%H_2O, 20%MeOH, 0.3%CH_3COOH and mobile phase B (pH 3.30): 99.7%MeOH, 0.3%CH_3COOH. The gradient programme was as follows: 0min-100%A, 17min-41.3%A, 17.2min-0%A, 20.2min-0%A, 20.3min-100%A, 34.0min-100%A. An injection volume of 20 µL was used, the column was maintained at 30 °C and the temperature of the sample manager was 4 °C. The flow rate was 0.04 mL min^{-1}, which gave an initial pressure of ~6500 psi.

A triple quadrupole mass spectrometer (TQD, Waters, UK) was used as described above. The mobile phase flow rate of 0.04 mL min^{-1} was directly introduced in the ion source without splitting. A detailed discussion of the methods and their validation is presented elsewhere (Tab. 5) (Baker and Kasprzyk-Hordern 2011).

Although due to experimental set-up two methods were used for quantification and enantiomeric profiling full method validation and robust analytical protocols ensured high performance of the overall analytical process.

3. Results and discussion

3.1. Amphetamine and methamphetamine

Estimation of amphetamine and methamphetamine abuse in communities with the usage of the *sewage epidemiology* approach has been undertaken by several research groups (Chiaia, Banta-Green et al. 2008; Zuccato, Chiabrando et al. 2008; Kasprzyk-Hordern, Dinsdale et al. 2009; Metcalfe, Tindale et al. 2010; Postigo, de Alda et al. 2010; Terzic, Senta et al. 2010; van Nuijs, Mougel et al. 2011) but possibility of overestimation of abuse trends (due to e.g. medical usage of amphetamine and methamphetamine and their metabolic formation) was also emphasized (Kasprzyk-Hordern, Dinsdale et al. 2009; Postigo, de Alda et al. 2010; van Nuijs, Castiglioni et al.

2010; Chiaia-Hernandez, Banta-Green et al. 2011). There are currently no solutions to overcome the above mentioned problems in order to provide more accurate estimates. However, enantiomeric profiling of amphetamine and methamphetamine might be of great importance here.

Amphetamine is a chiral compound with one asymmetric carbon. It can exist in the form of two enantiomers, which significantly differ in potency: *S*(+)-amphetamine has twice as high stimulant activity than *R*(-)-amphetamine (Kasprzyk-Hordern 2010). Amphetamine is the most commonly synthesized via the Leuckart method, which uses 1-phenyl-2-propanone and other reagents such as formic acid, ammonium formate or formamide to yield a racemic amphetamine. Another, less common, but stereoselective method involves reduction of appropriate diastereoisomers of norephedrine or norpseudoephedrine (King 2009). Both *S*(+)- and *S*(+)/*R*(-)-amphetamine are prescription medications. Amphetamine can also be excreted as a result of metabolism of methamphetamine and certain prescription drugs. For example, *R*(-)-amphetamine is excreted (alongside *R*(-)-methamphetamine) as a result of administration of selegiline (marketed as R(-)-enantiomer). *S*(+)-Amphetamine is formed as a result of an administration of clobenzorex. On the other hand metabolism of famprofazone leads to the formation of *S*(+)/*R*(-)-amphetamine and *S*(+)/*R*(-)-methamphetamine. Similarly, administration of fenproporex leads to the formation of *S*(+)/*R*(-)-amphetamine. Benzphetamine metabolism results in the formation of *S*(+)-amphetamine (and *S*(+)-methamphetamine) (Cody 2002). Furthermore, metabolism of amphetamine is known to be stereoselective: *S*(+)-amphetamine metabolises faster than *R*(-)-enantiomer.

Methamphetamine, similarly to amphetamine, contains one chiral carbon and exists in the form of two enantiomers, which differ in their psychostimulant effects with *S*(+)-enantiomer being much more active than *R*(-)-enantiomer. Methamphetamine, as opposed to amphetamine, is usually abused in pure enantiomeric form of the *S*(+)-enantiomer. However, also *R*(-)-enantiomer and the mixture of two enantiomers of methamphetamine can be abused (Nagai, Matsushima et al. 2000). Methamphetamine is the most commonly produced in clandestine laboratories by reduction of *1R,2S*(-)-ephedrine or *1S,2S*(+)-pseudoephedrine (naturally produced by *ephedra*). *R*(-)-enantiomer of methamphetamine can be produced by reduction of synthetic *1S,2R*(+)-ephedrine or *1R,2R*(-)-pseudoephedrine. Both the Leuckart route and reductive amination of 1-phenyl-2-propanone (P2P) are less commonly used and yield a racemic mixture of methamphetamine (King 2009). Enantiomeric ratios of methamphetamine and amphetamine are therefore closely related to configuration of precursors used for their synthesis, and this can provide useful information concerning the origins and synthetic methods used for illicit manufacture (Lee, Yang et al. 2007). Methamphetamine shares the same pharmacokinetic profile as amphetamine. This means that *S*(+)-methamphetamine metabolises faster in humans than *R*(-)-enantiomer, which leads to enrichment of excreted methamphetamine with *R*(-)-enantiomer (Levine 2003). Methamphetamine, similarly to amphetamine, can be used for valid medical treatment and can be excreted as a result of metabolism of certain drugs such as selegiline (*R*(-)-methamphetamine), benzphetamine (*S*(+)-methamphetamine) famprofazone (S(+)/*R*(-)-methamphetamine).

Due to different uses of amphetamine and methamphetamine (both medical and illicit) verification of their abuse through biological specimen analysis (usually urine and blood) is difficult. Enantiomeric analysis of abused amphetamines, their metabolites and precursors in biological specimen can help, as it can confirm or contradict the stated (meth)amphetamine sources of these compounds. The following information is usually taken into account:

1. Medical use of amphetamine and methamphetamine

As mentioned above, *S*(+)-methamphetamine, *S*(+)-amphetamine or *S*(+)/*R*(-)-amphetamine could be used for valid medical treatment. All of the above formulations are controlled substances, but are available for prescription use (this is not true for *S*(+)/*R*(-)-methamphetamine) (Cody and Schwarzhoff 1993; Cody 2002). Amphetamine is used in narcolepsy, attention deficit disorder in

children and short term weight loss. Methamphetamine is prescribed to treat attention deficit disorder in children and exogeneous obesity. In the USA methamphetamine is prescribed as *S*(+)-enantiomer. However, *R*(-)-methamphetamine is present in the Vicks Inhaler (an over-the-counter medication). On the other hand amphetamine can be prescribed as *S*(+)-enantiomer or racemate. In this case, interpretation of the enantiomeric composition depends on the composition of prescribed drug. If *S*(+)-amphetamine is prescribed, only *S*(+)-enantiomer should be found in urine. Similarly, if both enantiomers were used, there should be evidence of the appropriate metabolic profile of these enantiomers (Cody and Schwarzhoff 1993; Cody 2002).

2. Amphetamine and methamphetamine as metabolites

A number of drugs can lead to metabolic formation of amphetamine and methamphetamine. Among them are: amphetaminil, benzphetamine, clobenzorex, deprenyl, dimethylamphetamine, ethylamphetamine, famprofazone, fencamine, fenethylline, fenproporex, furfenorex, mefenorex, mesocarb and prenylamine. They are comprehensively reviewed by Cody (Cody 2002). Many precursors of illicit drugs belong to the group of anorectics but are often used for treatment of obesity (Liu and Liu 2002). In order to distinguish between (meth)amphetamine abuse and its formation due to use of prescription medication leading to the formation of (meth)amphetamine, the following factors are usually taken into account:

- Enantiomeric configuration of illicit drug
  Enantiomeric configuration influences the metabolism of amphetamine, methamphetamine and the precursor drugs. When racemic amphetamine and methamphetamine are administered, the *S*(+)-enantiomer is metabolised more rapidly than the *R*(-)-enantiomer. As a result, (meth)amphetamine in urine will be enriched with *R*(-)-enantiomer and the ratio of *R*(-)-to *S*(+)-(meth)amphetamine will be increasing over the course of the metabolism process (Cody 2002; Wang, Wang et al. 2005).
- Amphetamine to methamphetamine ratio
  The proportion of amphetamine and methamphetamine is also an important factor and can provide valuable information in the interpretation of laboratory results (Cody 2002).
- Detection of parent compounds and/or their unique metabolites
  For those precursor compounds that are excreted unchanged, detection of parent compounds or their unique metabolites, when related to the amount of methamphetamine and/or amphetamine present, can be a powerful tool to help properly interpret analytical data and evaluate the origin of studied illicit drugs (Cody 2002).
- Influence of pH
  Urinal pH can significantly affect the excretion patterns of amphetamines, which can complicate interpretation of metabolism data. Because amphetamines are basic drugs reabsorption in the kidneys is insignificant under acidic urine conditions, which results in the excretion of a higher percentage of parent drugs. Under alkaline conditions more significant reabsorption occurs, with a net effect of increased drug half-life and increased metabolic degradation. Although urine pH does not cause differential excretion of *S*(+) and *R*(-)-enantiomes, its effects on reabsorption rate will have a secondary effect on the observed enantiomeric composition (Liu and Liu 2002).

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As discussed above, this wide usage of different forms of amphetamine and methamphetamine and their stereoselective metabolism make verification of medical/illicit amphetamine use complex in biological specimens such as urine or blood. This process becomes even more difficult if verification is to take place in wastewater which provides only collective information on the whole population served by targeted WWTP rather than one individual.

In this study amphetamine quantified in wastewater was enriched with *R*(-)-enantiomer (Fig. 1). Enantiomeric fractions varied from 0.52 to 0.84, with median value of 0.64. This situation is expected in urine and subsequently in raw wastewater (obviously if no changes of *EFs* in sewers take place) if racemic amphetamine is administered. This is because, as was discussed earlier, *S*(+)-amphetamine metabolizes faster in humans than *R*(-)-amphetamine. Indeed, amphetamine is usually abused as racemate. Furthermore, according to NHS statistics in England, only *S*(+)-amphetamine is prescribed (dexamfetamine and lisdexamphetamine: 25 and 0.01 kg/2010) (NHS, 2010). These are relatively low levels when compared to illegal use (estimated usage of amphetamine and methamphetamine: 4.0 tonnes in 2003/2004; calculated with reference to seizures, purity and survey-based estimates of usage) (Singleton et al., 2006). Amphetamine can also be excreted in different enantiomeric forms as a result of metabolism of methamphetamine and certain prescription drugs (e.g. selegiline, prescription in England: 13 kg/2010 (NHS, 2010), which leads to the metabolic formation of *R*(-)-amphetamine and *R*(-)-methamphetamine). Again, these quantities are insignificant when compared to actual estimated abuse of amphetamine and methamphetamine.

The results of this study indicated that methamphetamine was only quantified in the August sampling campaign in three WWTPs at levels not exceeding 0.08 g/day (concentration, <1.8 ng/L). It was found to be enriched with *S*(+)-enantiomer in the case of WWTP 2 and 4 and was racemic in WWTP 1 (Fig. 2). Currently, there is no medical use of methamphetamine in England. Therefore, if present in wastewater it is thought to result from its abuse or metabolism of other drugs. As mentioned above, prescription drug selegiline (England: 13 kg/2010 (NHS, 2010)) can lead to the metabolic formation of *R*(-)-methamphetamine. If the latter is the case, only *R*(-)-enantiomer of methamphetamine should be found in wastewater. Although the limited data obtained in this study does not allow for accurate interpretation of methamphetamine usage trends in the studied WWTPs, it can be concluded that methamphetamine found in wastewater might result from abuse of mainly *S*(+)-methamphetamine with contribution from illicit *R*(-)/*S*(+)-methamphetamine and/or *R*(-)-methamphetamine, a metabolite of selegiline. The latter is less likely due to low usage of this pharmaceutical in the UK, although it cannot be excluded without having data on prevalence of selegiline use in the local area.

The above study, although limited in scope, indicates that enantiomeric analysis of amphetamine and methamphetamine might significantly contribute to more accurate estimation of their abuse patterns via *sewage epidemiology*. After taking into consideration medical use of (meth)amphetamine (this data can be obtained in most countries) and their possible metabolic formation from certain prescription drugs (this data can be also obtained in most countries), enantiomeric profiling might give invaluable information on enantiomeric purity of (meth)amphetamine being abused and might also indicate patterns of their abuse. The analysis of wastewater could also be critical in evaluating illicit distribution of different batches of drugs (and their spread) in local communities.

3.2. Hallucinogenic amines: MDMA and MDA

Hallucinogenic amines: MDMA and MDA have one asymmetric carbon centre and therefore they can exist in the form of two enantiomers, which differ both quantitatively and qualitatively in pharmacological activity: *S*(+)-enantiomers are more amphetamine-like stimulants and *R*(–)-enantiomers are more hallucinogenic (Fantegrossi 2008). MDMA's enantiomers have different serotonin (5-HT) neurotoxicity: *S*(+)-MDMA is a more potent neurotoxin than *R*(-)-MDMA. On the other hand, both isomers of MDA cause long-term serotonin neurotoxicity (Moore, Mozayani et al. 1996). It is however believed that much of the neurotoxicity of MDMA results from MDA, its more potent neurotoxic metabolite (Levine 2003).

There are four principal precursors, which can be used in manufacture of MDMA and related drugs: safrole, isosafrole, piperonal, 3,4-methylenedioxyphenyl-2-propanone (PMK). Many illicit

syntheses start with PMK and use either the Leuckart route or various reductive aminations. All of these methods produce racemic MDMA (King 2009). *S*(+)-MDMA is however known to undergo preferential metabolism over *R*(-)-MDMA, which leads to enrichment of MDMA with *R*(-)-enantiomer and preferential formation of *S*(+)-MDA. Moore et al. (Moore, Mozayani et al. 1996) observed that both primary routes of excretion in human (bile and urine) had greater concentrations of *R*(-)-MDMA than the *S*(+) isomer (*EF* of 0.57, autopsy findings). These fluids also contained twice the concentration of *S*(+)-MDA than the *R*(-)-isomer (*EF*=0.37, autopsy findings) as reported by Moore at al. (Moore, Mozayani et al. 1996). This is very important information, which allows for the verification of whether drug residue present in wastewater results from its actual consumption (*EF*>0.5) or direct disposal (*EF*=0.5). As MDMA does not currently have medical applications its presence in biological specimen is believed to result from its abuse.

The results of this study indicate that MDMA is found in raw wastewater in the form enriched with *R*(-)-MDMA. Mean *EF* value for raw wastewater collected during 5 sampling months and at 7 different WWTPs was found to be 0.68 (Fig. 3). This correlates well with published data on metabolism of MDMA in humans (Moore, Mozayani et al. 1996) and suggests that MDMA found in studied WWTPs results from MDMA abuse rather than direct disposal. It has to be however remembered that the uncertainty of such assumptions is probably high due to the influence of several parameters (such as different metabolism patterns in humans: age, gender, route of administration and co-administration with other drugs) on the overall *EF* of MDMA in wastewater, which should be taken into account and investigated further.

MDA, similarly to MDMA, has no medical usage and is synthesized and abused in racemic form. Similarly to MDMA, *S*(+)-MDA is preferentially metabolized to *R*(-)-MDA leading to enrichment of excreted MDA with *R*(-)-enantiomer (Meyer, Peters et al. 2009). However, if the presence of MDA in urine is expected due to MDMA abuse and not direct MDA use, an enrichment of MDA with *S*(+)-enantiomer should be expected in urine. It is worth remembering that in such cases, almost twice as much *S*(+)-MDA will be excreted in urine as compared to *R*(-)-MDA (Levine 2003). The above information may be of vital importance in sewage epidemiology as it potentially allows for the evaluation of the origin of MDA through making a distinction between actual abuse of MDA (*EF*>0.5) or metabolic formation of MDA as a result of MDMA use (*EF*<0.5).

In this study, MDA was only quantified in the August sampling campaign and it was found in raw wastewater to be enriched with *S*(+)-enantiomer, which suggests that its presence might be associated with MDMA abuse and not intentional MDA use (Fig. 4). The ratio of MDMA to MDA denoted: 9.8, 14.1, 6.0 in the case of WWTP 1, 2 and 3 respectively. The presence of MDA (alongside MDMA) in wastewater was reported by several groups (Castiglioni, Zuccato et al. 2006; Chiaia, Banta-Green et al. 2008; Huerta-Fontela, Galceran et al. 2008; Bijlsma, Sancho et al. 2009). Ratios between MDMA and MDA concentrations were in the range: 0.4 – 15. Furthermore, metabolism of MDMA was reported to lead to urinary excretion of 65% of the dose as parent drug and 7% as MDA (Baselt 2004), which gave an MDMA to MDA ratio of 9.3. This suggests, although with high uncertainty, that in the discussed study MDA found in wastewater is likely to result rather from MDMA abuse than MDA use. It should be however noted here that MDA can be also formed as a result of metabolism of another abused drug, MDEA. Although not discussed in this manuscript, MDEA was also a subject of investigation in this study and was not quantified in any of the analysed samples.

Enantiomeric analysis of hallucinogenic amines such as MDMA and MDA, which are distributed as racemates and have no medical use, can therefore provide simple and straightforward verification of their abuse: it can help in the differentiation between drugs present in wastewater which are derived from either their abuse or metabolic formation from different drugs, as well as allowing for distinction between their consumption and direct disposal.

3.3. Sympathomimetic amines: ephedrine and pseudoephedrine

Ephedrine has two chiral carbons and can therefore exist in the form of two pairs of enantiomers: *1R,2S*(-)-ephedrine, *1S,2R*(+)-ephedrine, *1S,2S*(+)-pseudoephedrine and *1R,2R*(-)-pseudoephedrine, which significantly differ in CNS system potency. The ephedrine enantiomers have a diastereomeric relationship with pseudoephedrine enantiomers. Only two diastereomers: *1R,2S*(-)-ephedrine and *1S,2S*(+)-pseudoephedrine are found in natural sources such as ephedra. It has to be however emphasized that the complementary enantiomers can be synthesised synthetically. *1R,2S*(-)-Ephedrine is used as a bronchodilator to treat bronchospasm associated with asthma, bronchitis and emphysema. It is also abused for its stimulant properties; however, its potency as a stimulator of the CNS is not as high as that of amphetamine. *1S,2S*(+)-Pseudoephedrine has the weakest CNS effects of this class of drugs and is primarily used as a decongestant and is present in over-the counter cold and allergy medications in combination with antihistamines and analgesics (Levine 2003).

Ephedrine is a prescription drug and it is also present in over-the-counter medications. Due to its high medical usage and also abuse it is commonly identified in wastewater (Postigo, de Alda et al. 2008; Postigo, de Alda et al. 2010; Baker and Kasprzyk-Hordern 2011). In this study, cumulative concentrations of ephedrine/pseudoephedrine found in raw wastewater varied from 8 ng/L to 1.6 µg/L (daily loads: 0.2 – 73 g/day) (Fig. 5). Unfortunately achiral analytical methodology, which is currently commonly used for quantification of ephedrine/pseudoephedrine (Chiaia, Banta-Green et al. 2008; Postigo, de Alda et al. 2008; Baker and Kasprzyk-Hordern 2011) does not allow for its enantiomeric profiling and as a result does not provide information on actual stereoisomer used. This lack of enantiomeric profiling therefore leads to an inability to determine the actual potency of ephedrine/pseudoephedrine used.

The results of this study indicate that out of the two enantiomers of ephedrine (*1S,2R*(+)- and *1R,2S*(-)-) and two enantiomers of pseudoephedrine (*1S,2S*(+)-and *1R,2R*(-)-), only natural *1R,2S*(-)-ephedrine and *1S,2S*(+)-pseudoephedrine were frequently detected in raw wastewater. *1S,2R*(+)-Ephedrine was detected at low levels in only three WWTPs during the August sampling campaign (*EFs* = 0.95, 0.96, 0.81 in WWTP 3, 4 and 6). The achiral analysis of ephedrine/pseudoephedrine indicated that ephedrine/pseudoephedrine was present in raw wastewater at daily loads exceeding 70 g/day, with the highest loads observed during winter months (Fig. 5). Furthermore, the analysis of diastereomeric fractions of *1R,2S*(-)-ephedrine and *1S,2S*(+)-pseudoephedrine in raw wastewater revealed that over the winter months ephedrine was enriched with *1S,2S*(+)-pseudoephedrine (Fig. 5). This is possibly due to higher usage of over-the-counter medications (containing *1S,2S*(+)-pseudoephedrine) for the treatment of mild symptoms of cold and contributing to higher concentrations of observed cumulative ephedrine/pseudoephedrine loads. During the spring and summer months the reverse situation was observed as ephedrine/pseudoephedrine was found to be enriched with a much more potent stimulant, *1R,2S*(-)-ephedrine. Cumulative loads of *1R,2S*(-)-ephedrine, *1S,2S*(+)-pseudoephedrine and total ephedrine (*1R,2S*(-)-ephedrine + *1S,2S*(+)-pseudoephedrine) for all WWTPs during different sampling campaigns are presented in Fig. 6. It can be observed that *1S,2S*(+)-pseudoephedrine reaches its peak load in February (170 g/day), which gradually decreases over March and April to 41 g/day in August. On the other hand *1R,2S*(-)-ephedrine was observed at the lowest levels in winter months (January March: 16-11 g/day) with a significant increase in loads in August (39 g/day). These findings were, as mentioned above, accompanied by a decrease of cumulative concentration of ephedrines throughout the sampling campaign between months: Feb – August. This is a very important finding indicating that non-enantioselective measurement of ephedrine concentrations cannot be a reliable indicator of actual potency of ephedrines used.

4. Conclusions

This manuscript is, to the authors' knowledge, the first to report the importance of enantiomeric profiling of abused drugs in *sewage epidemiology*. Enantiomeric profiling can supplement epidemiology data with valuable information on abuse trends and potency of chiral drugs and can also help with distinguishing between legal and illicit use of drugs as well as providing an indication of actual consumption and direct disposal.

The verification of amphetamine and methamphetamine abuse trends through the analysis of sewage is difficult. This is because both amphetamine and methamphetamine have medical use (different in different countries) and can be excreted in urine due to metabolism of certain prescription drugs. While it is possible to distinguish between legal and illicit use of amphetamine and methamphetamine in legal cases when biological matrix from a suspect is analyzed, this might prove to be impossible in sewage due to the large number of individuals being considered. Despite this obstacle, enantiomeric profiling of amphetamine and methamphetamine in populations (e.g. at regional and national scale) for which prescription and sales data exist, can provide valuable information of trends of (meth)amphetamine abuse, e.g. whether enantiomerically pure form of abused drug or racemic form are preferably used.

Enantiomeric profiling proves to be invaluable in the verification of MDMA and MDA abuse, and particularly in making a distinction between MDA abuse and its formation due to metabolism of MDMA. It can also help with making a distinction between actual consumption and direct disposal of MDMA. Similarly, enantiomeric profiling allows for a distinction to be made between widely used decongestant *1S,2S*(+)-pseudoephedrine and more potent and more likely abused *1R,2S*(-)-ephedrine.

Further study is needed in order to address several uncertainties related to enantiomeric profiling as a tool in *sewage epidemiology*. Among the most pressing phenomena to study and verify are:

- Stereoselective disposition of drugs in the body, which can be affected by several parameters. Among them are: disease, ethnic differences, sex, age and lifestyle as well as co-administration of other drugs, pH of urine (which might lead to reabsorption e.g. of amphetamine, and as result lower concentrations of the drug in urine and higher metabolism of the drug), etc (Kasprzyk-Hordern 2010). Only limited data, obtained for a low number of individuals, exists regarding stereoselective metabolism of chiral drugs of abuse. More information is needed to provide statistically sound estimation of stereoselective disposition of drugs and the importance of possible factors influencing streoselective behaviour of drugs in the body.
- The possibility of chiral inversion of the enantiomers of amphetamine-like compounds, although unlikely, needs to be further investigated. This has not been reported before, and thus far has been considered not to take place. This phenomenon is for example common in the case of NSAIDs (Kasprzyk-Hordern 2010).
- The fate of chiral drugs in sewers needs to be studied, as processes taking place in sewers, due to the presence of microorganisms, might be stereoselective in nature. Stereoselectivity of amphetamine-like drugs (amphetamine, MDMA and ephedrines) was for example observed during wastewater treatment (Kasprzyk-Hordern et al 2010; Kasprzyk-Hordern and Baker, 2012).

In summary, enantiomeric profiling should be undertaken together with other analytical methods in order to provide a reliable assessment of drugs use in communities. For example the analysis of metabolites (especially these characteristic for a drug of concern), of which not all are chiral, is vital in the verification of legitimate/illicit use, route of administration and consumption/direct disposal of drugs. It would also be interesting to explore other techniques widely used in forensic science, which could provide additional information of drugs abuse

trends in communities. Isotope ratio mass spectrometry, amongst others, is an interesting example.

References:

Baker, D. R. and B. Kasprzyk-Hordern (2011). "Multi-residue analysis of drugs of abuse in wastewater and surface water by solid-phase extraction and liquid chromatography-positive electrospray ionisation tandem mass spectrometry." Journal of Chromatography A **1218**(12): 1620-1631.

Baker, D. R. and B. Kasprzyk-Hordern (2011). "Multi-residue analysis of drugs of abuse in wastewater and surface water by solid-phase extraction and liquid chromatography-positive electrospray ionisation tandem mass spectrometry." J Chromatogr A **1218**(12): 1620-1631.

Banta-Green, C. J., J. A. Field, et al. (2009). "The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal wastewater." Addiction **104**(11): 1874-1880.

Baselt, R. C. (2004). Disposition of toxic drugs and chemicals in man. Foster City, Calif., Biomedical Publications.

Bijlsma, L., J. V. Sancho, et al. (2009). "Simultaneous ultra-high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater." Journal of Chromatography A **1216**(15): 3078-3089.

Bones, J., K. V. Thomas, et al. (2007). "Using environmental analytical data to estimate levels of community consumption of illicit drugs and abused pharmaceuticals." Journal of Environmental Monitoring **9**(7): 701-707.

Buechler, J., M. Schwab, et al. (2003). "Enantioselective quantitation of the ecstasy compound (R)- and (S)-N-ethyl-3,4-methylenedioxyamphetamine and its major metabolites in human plasma and urine." Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences **793**(2): 207-222.

Castiglioni, S., E. Zuccato, et al. (2006). "Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry." Analytical Chemistry **78**(24): 8421-8429.

Chiaia-Hernandez, A. C., C. J. Banta-Green, et al. (2011). "Interpreting methamphetamine levels in a high-use community." Environ Sci Pollut Res Int.

Chiaia, A. C., C. Banta-Green, et al. (2008). "Eliminating Solid Phase Extraction with Large-Volume Injection LC/MS/MS: Analysis of Illicit and Legal Drugs and Human Urine Indicators in US Wastewaters." Environmental Science & Technology **42**(23): 8841-8848.

Cody, J. T. (2002). "Precursor medications as a source of methamphetamine and/or amphetamine positive drug testing results." Journal of Occupational and Environmental Medicine **44**(5): 435-450.

Cody, J. T. and R. Schwarzhoff (1993). "INTERPRETATION OF METHAMPHETAMINE AND AMPHETAMINE ENANTIOMER DATA." Journal of Analytical Toxicology **17**(6): 321-326.

Daughton, C. G. (2001). Illicit Drugs in Municipal Sewage: Proposed New Nonintrusive Tool to Heighten Public Awareness of Societal Use of Illicit/Abused Drugs and Their Potential for Ecological Consequences. *Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues*. C. Daughton and T. Jones-Lepp. Washington, D.C., American Chemical Society. **ACS Symposium Series 791:** 348-364.

Fantegrossi, W. E. (2008). "In vivo pharmacology of MDMA and its enantiomers in rhesus monkeys." Experimental and Clinical Psychopharmacology **16**(1): 1-12.

Fornstedt, T., A. M. Hesselgren, et al. (1997). "Chiral assay of atenolol present in microdialysis and plasma samples of rats using chiral CBH as stationary phase." Chirality **9**(4): 329-334.

Harman, C., M. Reid, et al. (2011). "In situ calibration of a passive sampling device for selected illicit drugs and their metabolites in wastewater, and subsequent year-long assessment of community drug usage." Environ Sci Technol **45**(13): 5676-5682.

Huerta-Fontela, M., M. T. Galceran, et al. (2008). "Occurrence of psychoactive stimulatory drugs in wastewaters in north-eastern Spain." Science of the Total Environment **397**(1-3): 31-40.

Karolak, S., T. Nefau, et al. (2010). "Estimation of illicit drugs consumption by wastewater analysis in Paris area (France)." Forensic Science International **200**(1-3): 153-160.

Kasprzyk-Hordern, B. (2010). "Pharmacologically active compounds in the environment and their chirality." Chemical Society Reviews **39**(11): 4466-4503.

Kasprzyk-Hordern, B. (2010). "Pharmacologically active compounds in the environment and their chirality." Chem Soc Rev **39**(11): 4466-4503.

Kasprzyk-Hordern, B., R. M. Dinsdale, et al. (2009). "Illicit drugs and pharmaceuticals in the environment--forensic applications of environmental data. Part 1: Estimation of the usage of drugs in local communities." Environ Pollut **157**(6): 1773-1777.

Kasprzyk-Hordern, B., R. M. Dinsdale, et al. (2009). "Illicit drugs and pharmaceuticals in the environment - Forensic applications of environmental data. Part 1: Estimation of the usage of drugs in local communities." Environmental Pollution **157**(6): 1773-1777.

Kasprzyk-Hordern, B., V. V. R. Kondakal, et al. (2010). "Enantiomeric analysis of drugs of abuse in wastewater by chiral liquid chromatography coupled with tandem mass spectrometry." Journal of Chromatography A **1217**(27): 4575-4586.

King, L. A. (2009). Forensic chemistry of substance misuse. A guide to drug control, RSC.

Lai, F. Y., C. Ort, et al. (2011). "Refining the estimation of illicit drug consumptions from wastewater analysis: Co-analysis of prescription pharmaceuticals and uncertainty assessment." Water Res.

Lee, J. S., W. K. Yang, et al. (2007). "Monitoring precursor chemicals of methamphetamine through enantiomer profiling." Forensic Science International **173**(1): 68-72.

Levine, B. P. D. (2003). Principles of forensic toxicology. Washington, DC, AACC Press.

Liu, J. T. and R. H. Liu (2002). "Enantiomeric composition of abused amine drugs: chromatographic methods of analysis and data interpretation." Journal of Biochemical and Biophysical Methods **54**(1-3): 115-146.

Mari, F., L. Politi, et al. (2009). "Cocaine and heroin in waste water plants: A 1-year study in the city of Florence, Italy." Forensic Science International **189**(1-3): 88-92.

Metcalfe, C., K. Tindale, et al. (2010). "Illicit drugs in Canadian municipal wastewater and estimates of community drug use." Environ Pollut **158**(10): 3179-3185.

Meyer, M. R., F. T. Peters, et al. (2009). "Investigations on the human hepatic cytochrome P450 isozymes involved in the metabolism of 3,4-methylenedioxy-amphetamine (MDA) and benzodioxolyl-butanamine (BDB) enantiomers." Toxicology Letters (Shannon) **190**(1): 54-60.

Moore, K. A., A. Mozayani, et al. (1996). "Distribution of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) stereoisomers in a fatal poisoning." Forensic Science International **83**(2): 111-119.

Nagai, T., K. Matsushima, et al. (2000). "Interpretation and enantiomer analysis of methamphetamine abusers' urine and illegally brewed methamphetamine crystals." Journal of Analytical Toxicology **24**(2): 140-145.

Postigo, C., M. J. L. de Alda, et al. (2008). "Fully automated determination in the low nanogram per liter level of different classes of drugs of abuse in sewage water by on-line solid-phase extraction-liquid chromatography-electrospray-tandem mass spectrometry." Analytical Chemistry **80**(9): 3123-3134.

Postigo, C., M. J. L. de Alda, et al. (2010). "Drugs of abuse and their metabolites in the Ebro River basin: Occurrence in sewage and surface water, sewage treatment plants removal efficiency, and collective drug usage estimation." Environment International **36**(1): 75-84.

Postigo, C., M. J. Lopez de Ada, et al. (2008). "Analysis of drugs of abuse and their human metabolites in water by LC-MS(2): A non-intrusive tool for drug abuse estimation at the community level." Trac-Trends in Analytical Chemistry **27**(11): 1053-1069.

Terzic, S., I. Senta, et al. (2010). "Illicit drugs in wastewater of the city of Zagreb (Croatia) - Estimation of drug abuse in a transition country." Environmental Pollution **158**(8): 2686-2693.

van Nuijs, A. L., S. Castiglioni, et al. (2010). "Illicit drug consumption estimations derived from wastewater analysis: A critical review." Sci Total Environ.

van Nuijs, A. L., J. F. Mougel, et al. (2011). "Sewage epidemiology--a real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium." Environ Int **37**(3): 612-621.

van Nuijs, A. L. N., B. Pecceu, et al. (2009). "Can cocaine use be evaluated through analysis of wastewater? A nation-wide approach conducted in Belgium." Addiction **104**(5): 734-741.

Wang, S. M., T. C. Wang, et al. (2005). "Simultaneous determination of amphetamine and methamphetamine enantiomers in urine by simultaneous liquid-liquid extraction and diastereomeric derivatization followed by gas chromatographic-isotope dilution mass spectrometry." Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences **816**(1-2): 131-143.

Zuccato, E., C. Chiabrando, et al. (2008). "Estimating community drug abuse by wastewater analysis." Environmental Health Perspectives **116**(8): 1027-1032.

Zuccato, E., C. Chiabrando, et al. (2005). "Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse." Environ Health **4**: 14.

Table 1. Selected chiral drugs.

Name	**CAS**
R/S(±)-Amphetamine $C_9H_{13}N$	R(-): 300-62-9 S(+): 51-64-9 R/S(±): 300-62-9
R/S(±)-Methamphetamine $C_{10}H_{15}N$	R(-): 33817-09-3 S(+): 537-46-2
R/S(±)-MDA $C_{10}H_{13}NO_2$	4764-17-4
R/S(±)-MDMA $C_{11}H_{15}NO_2$	42542-10-9
1R,2S(-)-Ephedrine HCl $C_{10}H_{15}NO \cdot HCl$	50-98-6
1S,2R(+)-Ephedrine HCl $C_{10}H_{15}NO \cdot HCl$	24221-86-1
1S,2S(+)-Pseudoephedrine HCl $C_{10}H_{15}NO \cdot HCl$	345-78-8
1R,2R(-)-Pseudoephedrine $C_{10}H_{15}NO$	90-82-4
R/S(±)-Amphetamine-d11 $C_9H_2D_{11}N$	NA
R/S(±)-Methamphetamine-d14 $C_{10}HD_{14}N$	NA
R/S(±)-MDA-d5 $C_{10}H_8D_5NO_2$	136765-42-9
R/S(±)-MDMA-d5 $C_{11}H_{10}D_5NO_2$	136765-43-0

Table 2. General information on the studied WWTPs.

WWTP	Parameter		
	Population served (thousands)	**Flows [L s^{-1}]**	**Wastewater (% industrial/% domestic)**
WWTP 1	15	89-212	10-15/85-90
WWTP 2	10	32-75	10-15/85-90
WWTP 3	11	40-115	10-15/85-90
WWTP 4	190	366-1300	30/70
WWTP 5	240	603-1231	30/70
WWTP 6	244	476-1378	30/70
WWTP 7	190	395-563	20/80

Table 3. Optimised MRM conditions for the analysis of chiral drugs by UPLC/MS/MS.

Analyte	CV/CE	MRM1 (quantification)	CV/CE	MRM2 (confirmation)
R/S(±)-Amphetamine	18/8	136.16>119.10	18/16	136.16>91.10
1R,2S(-)/*1S,2R*(+)-Ephedrine/ *1S,2S*(+)/*1R,2R*(-)-Pseudoephedrine	23/12	166.09>148.10	23/21	166.09>133.00
R/S(±)-MDA	21/11	180.03>163.10	21/22	180.03>105.10
R/S(±)-MDMA	24/13	194.09>163.10	24/24	194.09>105.10
R/S(±)-Methamphetamine	24/10	150.20>119.05	24/19	150.20>91.10
R/S(±)-Amphetamine-d11	18/8	147.16>130.10	-	-
R/S(±)-MDA-d5	21/11	185.09>168.10	-	-
R/S(±)-MDMA-d5	26/13	199.1>165.10	-	-
R/S(±)-Methamphetamine-d14	24/19	164.16>98.10	-	-

CV-cone voltage [V]; CE-collision energy [eV]

Table 4. Validation parameters for SPE-Chiral LC-MS/MS method.

Analyte	**Method parameters**				
	EF_o*	**R_s**	**R^2****	**RSD%**	
				10 [ng/L]	**50 [ng/L]**
R/S(±)-Amphetamine (IS: *R/S*(±)-AMPH-d11)	0.52±0.02	2.1	0.999	6.5	3.7
1R,2S(-)-Ephedrine (IS: *R/S*(±)-MDMA-d5)	0.52±0.03***	1.2	0.998	3.6	5.2
1S,2S(+)-Pseudoephedrine (IS: *R/S*(±)-MDMA-d5)		2.3	0.999	8.7	6.8
R/S(±)-MDA (IS: *R/S*(±)-MDA-d5)	0.48±0.01	3.0	0.998	7.8	3.3
R/S(±)-MDMA (IS: *R/S*(±)-MDMA-d5)	0.48±0.01	1.9	0.999	7.5	2.6
R/S(±)-Methamphetamine (IS: *R/S*(±)-METH-d14)	0.47±0.01	0.95	0.999	4.9	3.7

*- EF_o – enantiomeric fraction in standard solution spiked with racemic chiral drug (concentrations: 1-500 ng/L)
**- Studied linearity range: 1-500ng/L
***- the value refers to *DF* (diastereomeric fraction) in standard solution spiked with *1R,2S*(-)-ephedrine and *1S,2S*(+)-pseudoephedrine
IS – Internal standard

Table 5. Validation parameters for SPE-UPLC-MS/MS method.

Analyte	**Method parameters**				
	Linearity range [ng/L]	**R^2**	**RSD%**		**MQL [ng/L]**
			0.5 [ng/L]	**50 [ng/L]**	
Amphetamine (IS: Amphetamine-d11)	0.5-1000	0.999	<MQL*	10	5.1
Ephedrine/ Pseudoephedrine (IS: Amphetamine-d11)	0.5-1000	0.999	<MQL	10.7	5.6
MDA (IS: MDA-d5)	0.1-1000	0.999	11	2.1	4.2
MDMA (IS: MDMA-d5)	0.1-1000	0.999	9.4	3.9	0.7
Methamphetamine (IS: Methamphetamine-d14)	0.05-1000	0.999	8.3	4.1	0.6

* - MQL – method quantification limit for WWTP influent
IS – Internal standard

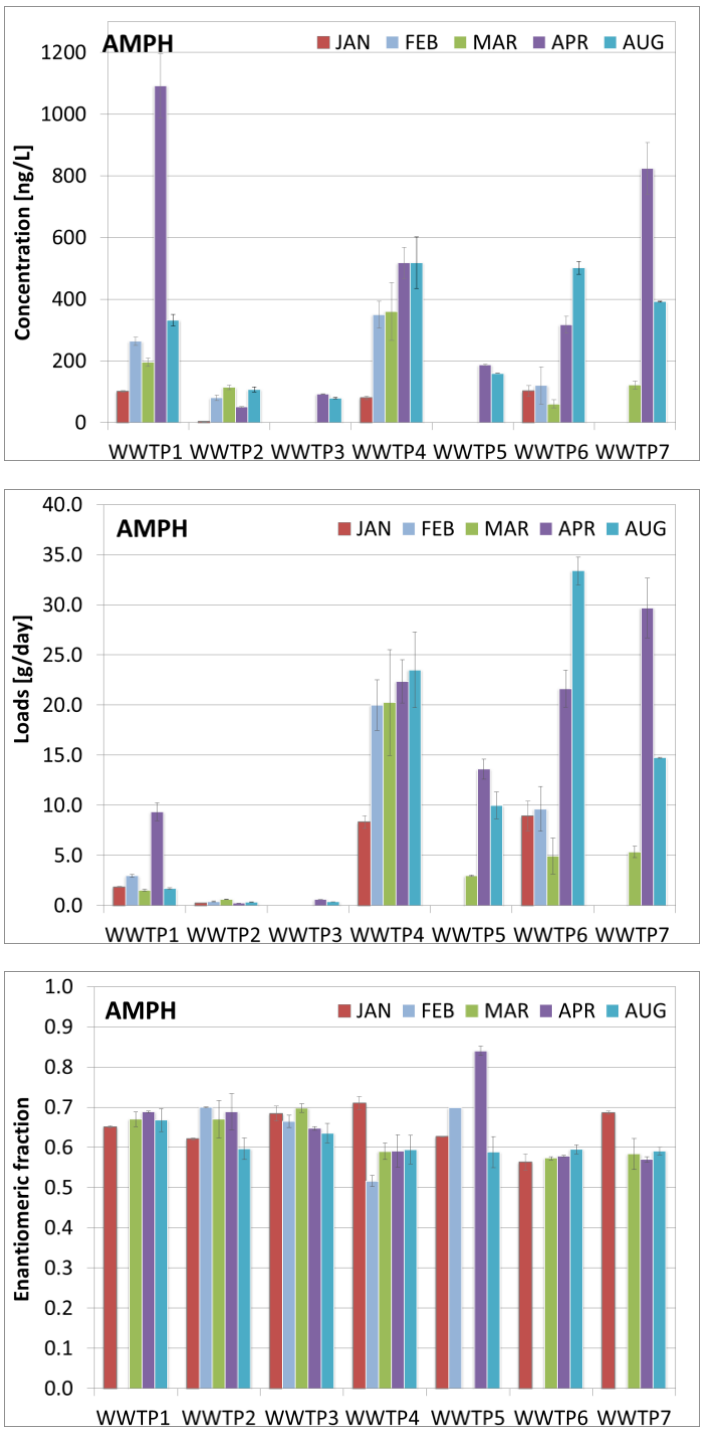


Fig. 1. Amphetamine in sewage: concentrations, loads and enantiomeric fractions.

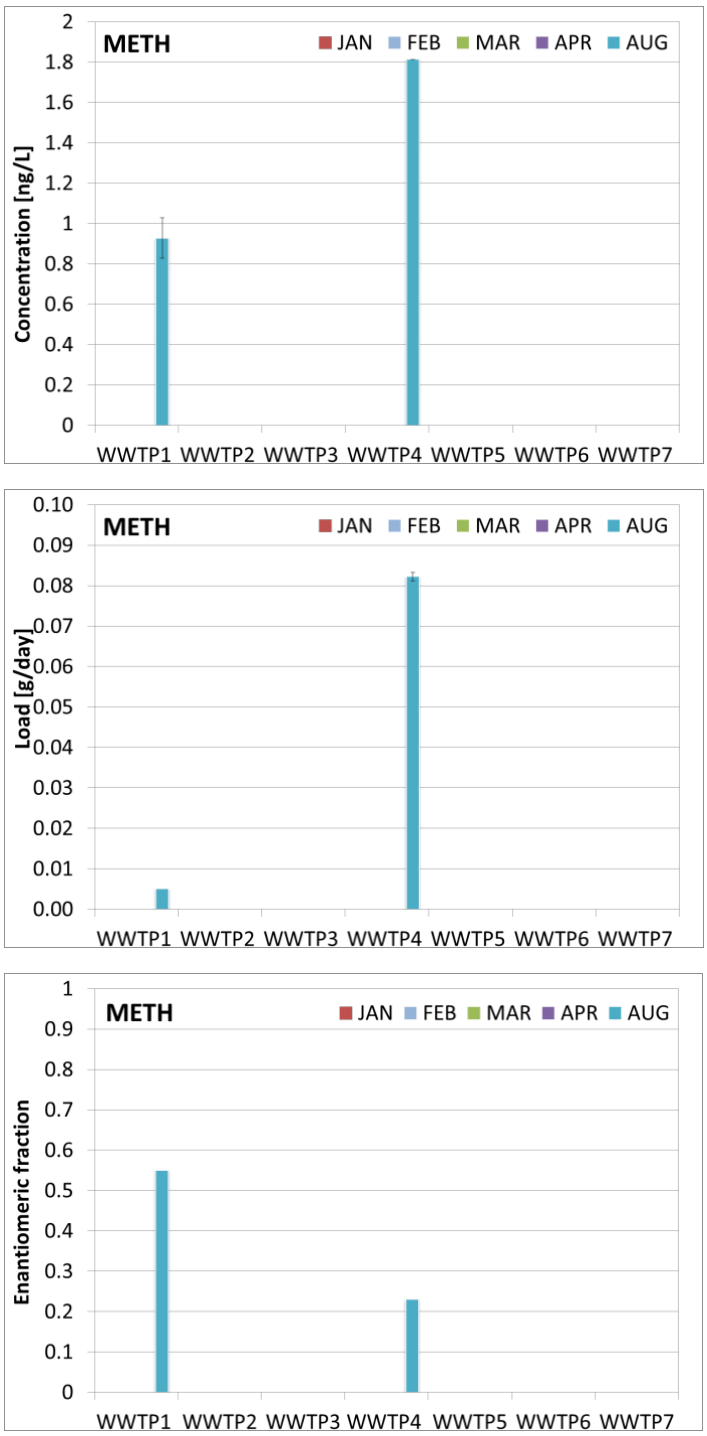


Fig. 2. Methamphetamine in sewage: concentrations, loads and enantiomeric fractions.

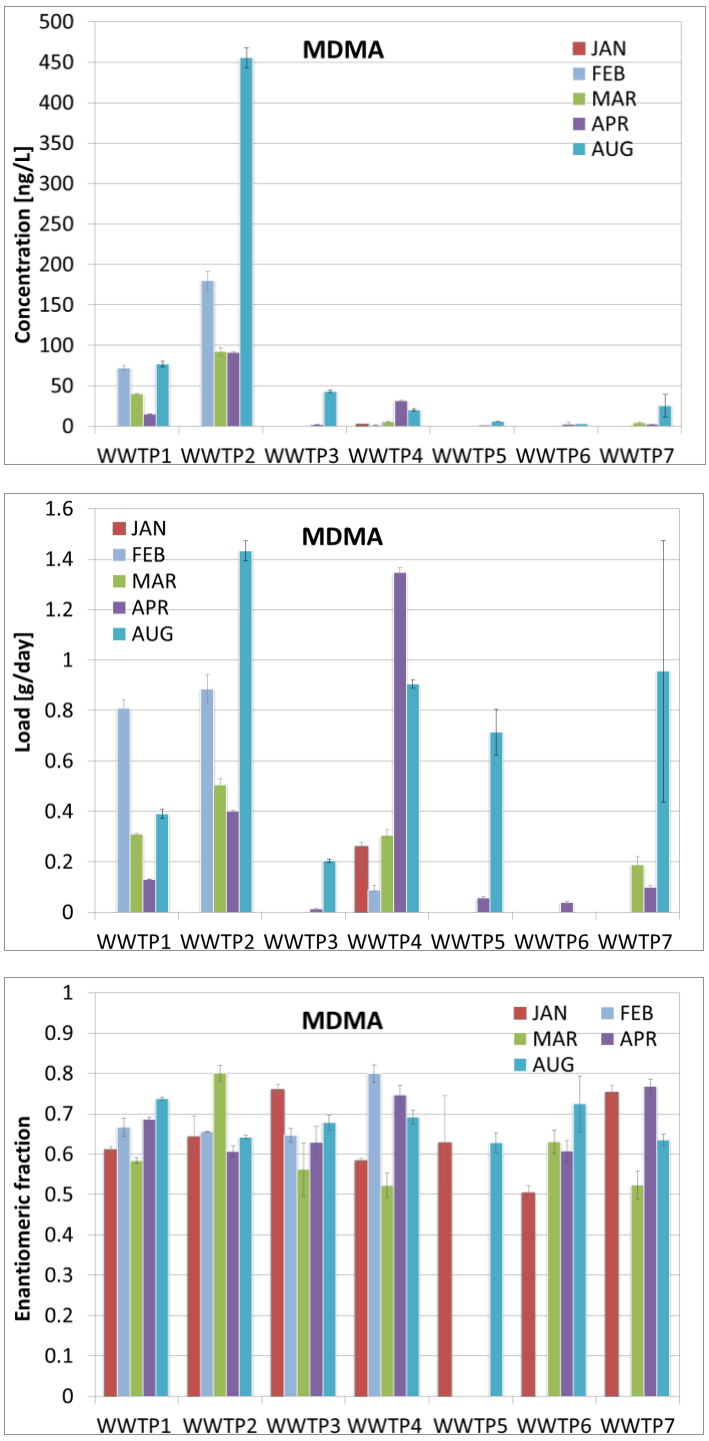


Fig. 3. MDMA in sewage: concentrations, loads and enantiomeric fractions.

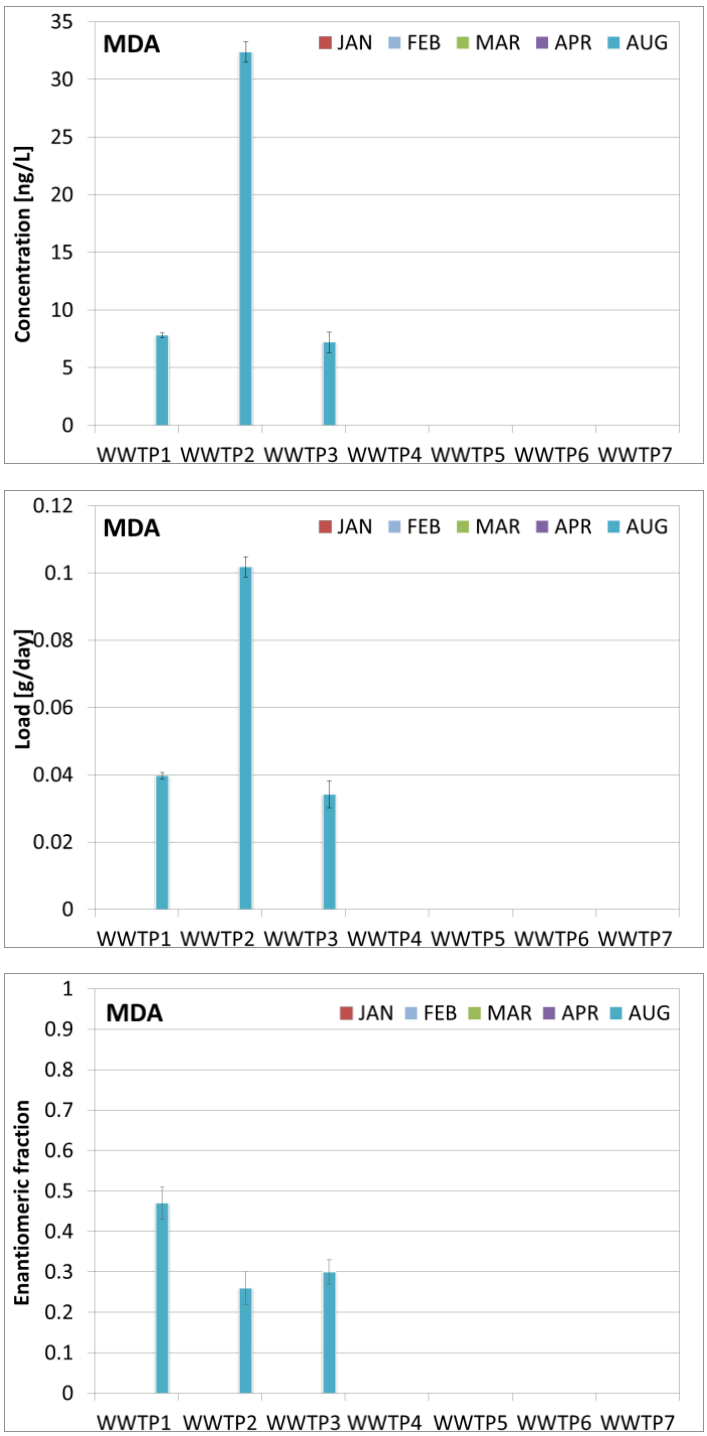
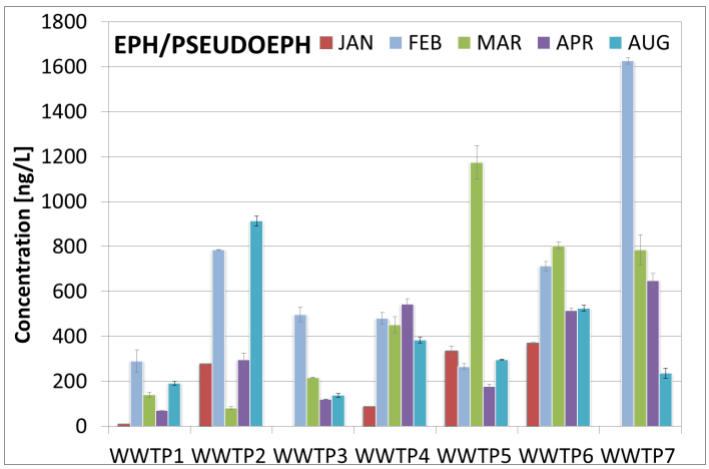
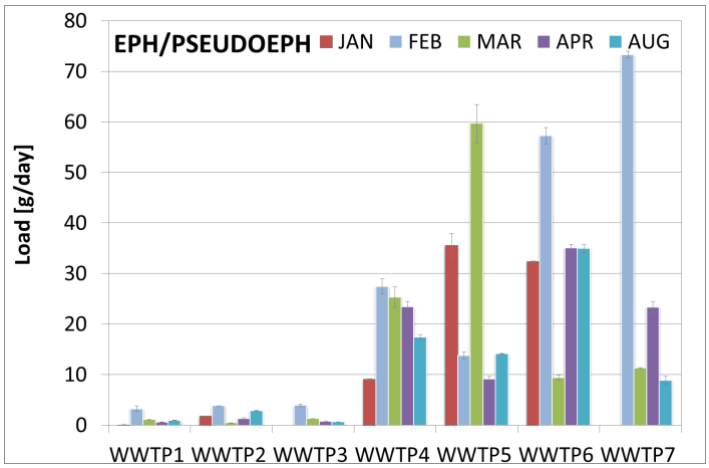


Fig. 4. MDA in sewage: concentrations, loads and enantiomeric fractions.





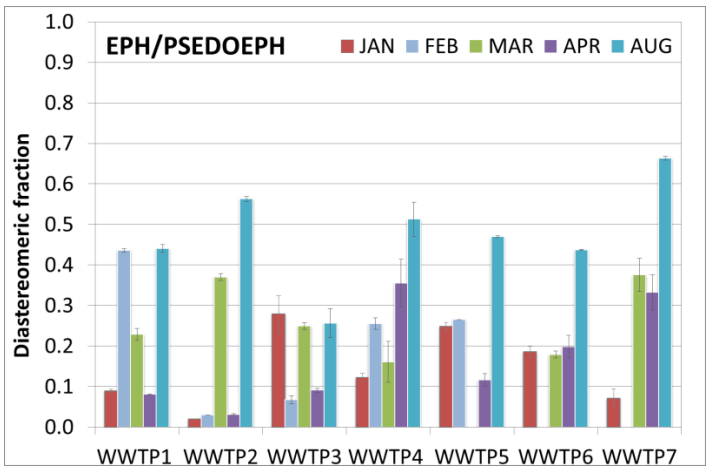


Fig. 5. Ephedrine in sewage: concentrations, loads and diastereomeric fractions.

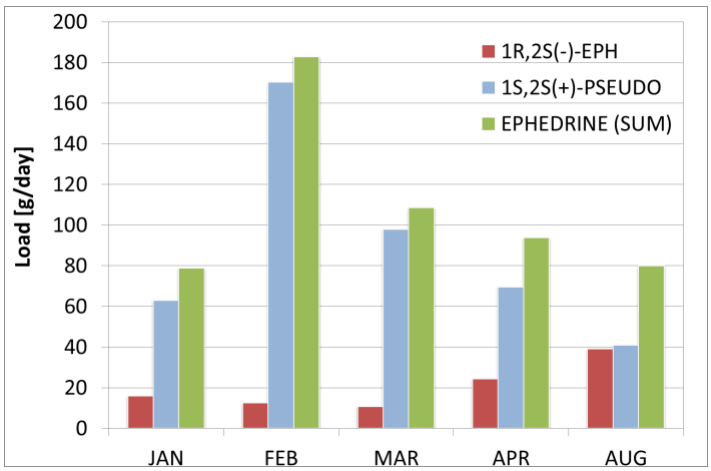


Fig. 6. *1R,2S*(-)-Ephedrine and *1S,2S*(+)-pseudoephedrine loads in sewage (sum of loads from all WWTPs).