DELIVERABLE 2.1-2.4

D2.1 Report on methods for specific health-related biomarkers in sewage

D2.2 Report on methods for enantioselective analysis and biosensors

D2.3 Report on multichannel biosensors for human biomarkers

D2.4 Report on analytical tools for new drugs/psychoactive compounds and their transformation products in sewage

Grant Agreement number: 317205

Project acronym: SEWPROF

Project title: A new paradigm in drug use and human health risk assessment: Sewage profiling at the community level

Marie Curie Initial Training Networks (ITN)

Call: FP7-PEOPLE-2012-ITN

PROJECT COORDINATOR: Dr Barbara Kasprzyk-Hordern, University of Bath

Project start date and duration: 1 October 2016, duration 48 months

Date of report: 09 July 2016

WP2 - Development of methods for targeted analysis and screening of biomarkers

WP2 aimed to provide new solutions to the analysis of very complex and heterogeneous environmental matrices through the development of sensitive and selective analytical techniques. New methodologies utilizing hyphenated (mass spectrometry-based) techniques were developed for targeted analysis, screening of unknowns and retrospective analysis of wastewater samples. More specifically, the main objectives were:

- To develop methods for specific health-related biomarkers in sewage;
- To develop methods for the enantiospecific analysis of biomarkers;
- To develop multichannel biosensors for biomarkers**;**
- To develop analytical tools for the identification, confirmation and quantification of new drugs/psychoactive compounds and their transformation products in sewage.

This report summarizes the deliverables related to WP2 highlighting the main research results obtained as publications in peer reviewed journals, and (when available) manuscripts in preparation.

The objectives of this WP were fulfilled within the projects conducted by ESRs/ERs.

The work performed in WP2 provides a strong and reliable analytical platform which has been further used in WP3 and WP4 for more specific epidemiological case-studies.

Deliverable	Direct contributors	DELIVERABLE TITLE[1]
D2.1	***ESR4, ESR5***	Methods for specific health-related biomarkers in sewage
D2.2	***ESR6***	Methods for enantioselective analysis
D2.3	***ER1***	Multichannel biosensors for human biomarkers
D2.4	***ESR7, ESR8, ESR9***	Analytical tools for new drugs/psychoactive compounds and their transformation products in sewage

D 2.1 Report on methods for specific health-related biomarkers in sewage

Analysis of biomarkers in sewage has been internationally studied and accepted as a complementary tool to traditional epidemiological methods. So far, the approach has mostly focused on the determination of legal or illegal drug use at the community level. However, there is large potential for wastewater-based epidemiology (WBE) approach to be extended to human health biomarkers for the assessment of community-wide health and disease. Among a range of candidate health biomarkers in sewage, oxidative stress biomarkers, F_2-isoprostanes (F_2-IsoPs) have gained attention as a prototype to study cumulative oxidative stress at a community level. These biomarkers have been studied in the frame of the project of **ERS4**. ESR4 has developed methods for the analysis of selected health biomarkers (e.g., oxidative stress: F2-isoprostanes, 8-hydroxydeoxyguanosine, allergy: antihistamines) using LC/MS techniques and qrt-PCR. In the same context, **ESR5** has developed specific analytical methods for the analysis of human biomarkers reflecting exposure to environmental and food toxic chemicals. Suitable biomarkers were selected as measurable substances in sewage and stable and specific indicators of toxic chemicals ingested or inhaled by a subject. The screening for these biomarkers has been done among the emerging and priority pollutants following the current literature and the priority lists from the Environmental Protection Agency.

Publication 1. Produced by free-radical catalysed oxidation from arachidonic acid, F_2-IsoPs have been accepted as a reliable indicator of total systematic oxidative stress. For the first time, 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), was quantitatively analysed in wastewater using an analytical method consisting of liquid chromatography–high resolution mass spectrometry coupled to immunoaffinity clean-up (IAC-LC-HRMS). Factors influencing the method's robustness were investigated, including analyte stability in sewage and enzymatic deconjugation with β-glucuronidase. The IAC-LC-HRMS method was linear over the range of 0.1–100 ng/mL with correlation coefficient (R^2) of 0.999. The quantification limits were sufficiently low to detect 8-iso-$PGF_{2\alpha}$ in sewage (method quantification limit of 0.3 ng/L) and precision, expressed as relative standard deviation was less than 7% and the accuracy expressed as relative recovery was in the 103–113% range. As a result, the application of the method to 24-h composite wastewater samples from Oslo showed 8-iso-$PGF_{2\alpha}$ concentrations of 18.9–23.3 ng/L for 8 days in March 2015. This study demonstrated a standard method to analyse 8-iso-$PGF_{2\alpha}$ in sewage that contributes to the further investigation of the potential use of 8-iso-$PGF_{2\alpha}$ as a sewage biomarker for assessing the status of community health.

Publication 2. To verify the hypothesis that the level of F_2-IsoPs is correlated with smoking and alcohol consumption, the most intensively studied F_2-IsoP isomer, 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) was analysed in wastewater by LC-MS coupled with immunoaffinity clean-up (IAC-LC-MS). Raw 24 hr-composite wastewater samples were collected from Norwegian and other European cities in 2014 and 2015. The patterns of 8-iso-$PGF_{2\alpha}$ in the different cities were then compared. Using the same samples, biomarkers of alcohol (ethyl sulfate) and tobacco (trans-3′-hydroxycotinine) consumption were also analysed for the investigation of possible correlation between 8-iso-$PGF_{2\alpha}$ and the legalised drugs.

Publication 3. This study presents the measurement of alcohol consumption in 20 cities across 11 countries through the use of WBE and reports the application of these data for the risk assessment of alcohol on a population scale using the margin of exposure (MOE) approach. Raw 24-h composite wastewater samples were collected over a one-week period from 20 cities following a common protocol. For each sample a specific and stable alcohol consumption biomarker, ethyl sulfate (EtS) was determined by liquid chromatography coupled to tandem mass spectrometry. The EtS concentrations were used for estimation of per capita alcohol consumption in each city, which was further compared with international reports and applied for risk assessment by MOE. The average per capita consumption in 20 cities ranged between 6.4 and 44.3 L/day/1000 inhabitants. An increase in alcohol consumption during the weekend occurred in all cities, however the level of this increase was found to differ. In contrast to conventional data (sales statistics and interviews), WBE revealed geographical differences in the level and pattern of actual alcohol consumption at an inter-city level. All sampled cities were in the “high risk” category (MOE < 10) and the average MOE for the whole population studied was 2.5. These results allowed direct comparisons of alcohol consumption levels,

patterns and risks among the cities. This study shows that WBE can provide timely and complementary information on alcohol use and alcohol associated risks in terms of exposure at the community level.

Publication 4. ESR5 has specific analytical methods for stable human biomarkers reflecting exposure to environmental and food toxicants, starting the selection from endocrine disruptors (pesticides, phytoestrogens or fungal toxins). The presence of the selected biomarkers in sewage was assessed using techniques suitable to identify trace amounts chemicals in environmental matrices with high specificity and sensitivity: LC-QqQ and LTQ-Orbitrap. Additionally, the stability of the human metabolites 3-PBA and cis- and trans-DCCA and of the three parent compounds permethrin, cypermethrin and cyfluthrin was investigated in influent wastewater.

Publications

1. Y Ryu, MJ Reid and KV Thomas: Liquid chromatography–high resolution mass spectrometry with immunoaffinity clean-up for the determination of the oxidative stress biomarker 8-iso-prostaglandin F2alpha in wastewater. *Journal of Chromatography A, 2015, 1409: 146-151.*

http://www.sciencedirect.com/science/article/pii/S0021967315010286

2. Y Ryu, E Gracia-Lor, MJ Reid, S Castiglioni, JG Bramness and KV Thomas: Analysis of oxidative stress biomarker 8-iso-prostaglandin $F_{2\alpha}$ in wastewater and its correlation with legalised drugs, *Manuscript in preparation, 2016*

3. Y Ryu, D Barceló, LP Barron, L Bijlsma, S Castiglioni, P de Voogt, E Emke, F Hernández, FY Lai, A Lopes, M López de Alda, N Mastroianni, K Munro, J O'Brien, C Ort, BG Plósz, MJ Reid, V Yargeau and KV Thomas: Comparative measurement and quantitative risk assessment of alcohol consumption through wastewater-based epidemiology: An international study in 20 cities, *Science of the Total Environment, 2016, 565: 977-983.*

http://www.sciencedirect.com/science/article/pii/S0048969716308312

4. NI Rousis, S. Castiglioni: Determination of biomarkers of exposure in raw wastewater by liquid chromatography-tandem mass spectrometry – Pesticides as the case study, *Manuscript in preparation, 2016*

Conferences

Y Ryu, MJ Reid and KV Thomas: Community health assessment by analysis of sewage biomarkers. Working Group Meetings COST Action ES1307, 28th October 2014, Malta

Y Ryu, MJ Reid and KV Thomas**:** Analysis of oxidative stress biomarker 8-iso-prostaglandin $F_{2\alpha}$ in wastewater by liquid chromatography-mass spectrometry coupled with immunoaffinity clean-up. 15thEuCheMS International Conference on Chemistry and the Environment, 22nd September 2015, Germany

Y Ryu, MJ Reid and KV Thomas: Analysis of oxidative stress biomarker 8-iso-prostaglandin $F_{2\alpha}$ in sewage and its correlation with legalised drugs. Testing the waters 2nd International Conference on «Wastewater-based drug epidemiology», 15th October 2015, Switzerland

NI Rousis et al. Determination of biomarkers of exposure in raw wastewater by liquid chromatography-tandem mass spectrometry – Pesticides as the case study. 2nd International Conference on «Wastewater-based drug epidemiology» - Testing the Waters, Monte Verita, Ascona, Switzerland, 11-15 October 2015

Y Ryu, D Barceló, LP Barron, LBijlsma, S Castiglioni, E Emke, F Hernández, A Lopes, M López de Alda, N Mastroianni, C Ort, BG Plósz, MJ Reid, P de Voogt, V Yargeau and KV Thomas: Measurement of alcohol consumption in a large multi-city study through sewage based epidemiology. Testing the Waters 2nd International Conference on Wastewater-based drug epidemiology, 12th October 2015, Switzerland.

D 2.2 Report on the development of methods for the enantiospecific analysis of biomarkers

A more exploratory part of WP2 was dedicated to the development of sensitive enantioselective methodologies for the analysis of chiral biomarkers and pharmacologically active compounds in liquid and solid sewage samples (work of **ESR6**). Since human pharmacokinetics shows stereoselectivity in the case of many chiral xenobiotics, analysis of human biomarkers at enantiomeric level can give invaluable information regarding pathways of human exposure to these compounds. ESR6 involved in this part has explored enantiomeric profiling in (i) estimation of (illicit) drug use via *sewage epidemiology* (e.g. for verification of their potency, origin, route of administration and monitoring of changing patterns of their use; distinction between legal and illicit use) and (ii) in the wider context of chiral pharmaceuticals analysis in sewage. Chiral-LC-MS-MS (QqQ and QTOF) were used for target analysis of chiral compounds, their metabolites and transformation by-products and screening of unknowns at enantiomeric level. SPE and MAE were used for the extraction of analytes from both liquid and solid matrices. The developed and validated methods were applied in WP4, the Europe-wide monitoring.

Publication 1. This paper proposes a novel multi-residue enantioselective method using a CBH (cellobiohydrolase) column, for the analysis of 56 drug biomarkers in wastewater. These are: opioid analgesics, amphetamines, cocaine, heroin, stimulants, anaesthetics, sedatives, anxiolytics, designer drugs, phosphodiesterase-5 (PDE5) inhibitors, amphetamine and methamphetamine drug precursors. Satisfactory enantiomeric separation was obtained for 18 pairs of enantiomers including amphetamine, methamphetamine, MDMA (3,4-methylenedioxy-methamphetamine) and its metabolites HMA (4-hydroxy-3-methoxyamphetamine) and HMMA (4-hydroxy-3-methoxy-methamphetamine), PMA (para-methoxyamphetamine), MDA ((±)- 3,4-methylenedioxyamphetamine) and mephedrone. The method was applied in a one week monitoring study of a large wastewater treatment plant in the UK. Most target drugs were found at quantifiable concentrations in analysed samples. Enantiomeric profiling revealed that amphetamine, methamphetamine and MDMA were found enriched with R-(-)-enantiomers, probably due to their stereoselective metabolism favouring S-(+)-enantiomers. MDA was either enriched with R-(-)- or S-(+)-enantiomer indicating that its presence might be due to either abuse of racemic MDA or abuse of racemic MDMA respectively. Non-racemic enantiomeric fractions were also observed in the case of HMMA and mephedrone, suggesting enantioselective metabolism. This is the first time that chiral separation and wastewater profiling of mephedrone, PMA, MDMA and its metabolites HMA and HMMA have been reported.

Publication 2. Determination of pharmacologically active chiral compounds (cPACs) at the enantiomeric level in environmental matrices yields vital information for improved WBE, development of more accurate environmental risk assessment, and improved understanding of cPAC fate in wastewaters and the environment. This review gives an up-to-date commentary on chiral liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS) to determine cPACs, including illicit drugs, in environmental matrices. Several applications are presented to demonstrate the benefits of performing environmental analysis of cPACs at the enantiomeric level. Finally, future perspectives in this rapidly developing field of research are outlined.

Publication 3. The issue of drug chirality is attracting increasing attention among the scientific community. The phenomenon of chirality has been overlooked in environmental research (environmental occurrence, fate and toxicity) despite the great impact that chiral pharmacologically active compounds (cPACs) can provoke on ecosystems. Therefore, special attention has been paid to the most recent advances in chiral analysis based on liquid chromatography coupled with mass spectrometry and the most popular protein based chiral stationary phases. Several groups of cPACs of environmental relevance, such as illicit drugs, human and veterinary medicines were discussed. The increase in the number of papers published in the area of chiral environmental analysis indicates that researchers are actively pursuing new opportunities to provide better understanding of environmental impacts resulting from the enantiomerism of cPACs.

Publications

1. **Castrignanò E**., Lubben A., Kasprzyk-Hordern B. Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry. Journal of Chromatography A, 1438: 84-99 (DOI: 10.1016/j.chroma.2016.02.015), Epub 2016 Feb 6, 2016.

http://www.sciencedirect.com/science/article/pii/S0021967316301017

2. Petrie B., Camacho-Munoz D., **Castrignanò E**., Evans S., Kasprzyk-Hordern B. Chiral Liquid Chromatography Coupled with Tandem Mass Spectrometry for Environmental Analysis of Pharmacologically Active Compounds LC GC EUROPE 28 (3), 151-160, 2015.

http://www.chromatographyonline.com/chiral-liquid-chromatography-coupled-tandem-mass-spectrometry-environmental-analysis-pharmacological

3. Camacho-Munoz D., Petrie B., **Castrignanò E**., Kasprzyk-Hordern B. Enantiomeric Profiling of Chiral Pharmacologically Active Compounds in the Environment with the usage of chiral Liquid Chromatography Coupled with Tandem Mass Spectrometry. Current Analytical Chemistry, 12, 2015.

http://benthamscience.com/journals/current-analytical-chemistry/article/135649/

Two further publications are planned on enantiomeric signature of chiral drugs in a pan-European study as well as chiral antibiotics.

Conferences:

1. **Castrignanò E**. and Kasprzyk-Hordern, B. Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry. In:Testing the Waters 2015: 2nd International Conference on Wastewater-based Drug Epidemiology, 2015-10-11 - 2015-10-15, Ascona, Switzerland.

2. Yang, Z., Kasprzyk-Hordern, B., Angls d'Auriac, M., Goggins, S., **Castrignanò E**., Rice, J., Thomas, K. V., Frost, C., Estrela, P., 2015. Community Sensors for Monitoring of Public Health by Means of Wastewater-Based Epidemiology. In: Testing the Waters 2015: 2nd International Conference on Wastewater-based Drug Epidemiology, 2015-10-11 - 2015-10-15, Ascona, Switzerland.

3. Kasprzyk-Hordern, B., **Castrignanò E**., Rydevik, A., Lopardo, L., Rice, J., Yang, Z. Wastewater-based epidemiology and future perspectives:testing urban water for community-wide public health assessment. In: 15th EuCheMS International Conference on Chemistry and the Environment, 2015-09-20 - 2015-09-25, Leipzig, Germany.

4. Yang, Z., Angls d'Auriac, M., Goggins, S., **Castrignanò E**., Rice, J., Estrela, P., Frost, C., Thomas, K.V., Kasprzyk-Hordern, B. Community Sensors for Monitoring Public Health using Wastewater-Based Epidemiology. In: 15th EuCheMS International Conference on Chemistry and the Environment, 2015-09-20 - 2015-09-25, Leipzig, Germany.

5. Yang, Z., Kasprzyk-Hordern, B., Angls d'Auriac, M., Goggins, S., **Castrignanò E**., Rice, J., Thomas, K. V., Frost, C., Estrela, P. Electrochemical Community Sensors for Monitoring of Public Health at Population Level Using WastewaterBased Epidemiology. In: The Fifteenth International Symposium on Electroanalytical Chemistry (15th ISEAC), 2015-08-13 - 2015-09-16, Changchun, China.

6. **Castrignanò E.** Wastewater-based epidemiology for community-wide antibiotics use assessment. Programme Working Group Meetings COST Action ES1307 "Sewage biomarker analysis for community health assessment", 27-28 October 2014, San Anton, Malta.

D2.3 Report on the development of multichannel biosensors for biomarkers

ER 1 has developed robust sensors capable of fast and reliable detection of low levels of analytes using portable instrumentation and simple (or no) sample preparation. Novel protein-binding DNA/PNA probes were used in combination with electrochemical techniques to develop a biosensor platform for monitoring of both protein and DNA biomarkers relevant to human health (e.g., bladder or prostate cancer) in sewage. ER1 has also developed novel chemistries and bio-immobilization techniques to enable the use of electrochemical biosensors for the detection of a variety of biomarkers; he has also optimized and validated the biosensors and demonstrated the operation of the system in the multichannel detection of multiple biomarkers. For DNA biomarkers, the targets were amplified by qrt-

PCR, while the performance of sensors was compared with standard biochemical techniques. The electrochemical data were validated by comparing model studies with gel electrophoresis experiments and LC-MS analysis of redox labelled probes.

Publication 1. For the first time, a novel quantitative community sewage sensor (namely DNA-directed immobilization of aptamer sensors, DDIAS) was developed for rapid and cost-effective estimation of cocaine use trends via WBE. Thiolated single-stranded DNA (ssDNA) probe was hybridized with aptamer ssDNA in solution, followed by co-immobilization with 6-mercapto-hexane onto the gold electrodes to control the surface density to effectively bind with cocaine. DDIAS was optimized to detect cocaine at as low as 10 nM with a dynamic range from 10 nM to 5 μM, which were further employed for the quantification of cocaine in wastewater samples collected from a wastewater treatment plant in seven consecutive days. The concentration pattern of the sampling week was comparable with that from mass spectrometry. The results demonstrated that the developed DDIAS can be used as community sewage sensors for rapid and cost-effective evaluation of drug use trends, and potentially implemented as a powerful tool for on-site and real-time monitoring of wastewater by un-skilled personnel.

Publication 2. A viewpoint was raised to indicate the need to develop novel analytical tools that are able to accurately and rapidly monitor low levels of biomarkers/pathogens with minimal sample processing by unskilled personnel at the site of sample collection. Emerging biosensing technology play a key role in the in situ quantitative analysis of biomarkers and pathogens in sewage due to rapid response times, low cost, minimal sample processing, high data resolution and ability to operate remotely. Community sewage sensors employed to detect biomarkers of health and diseases at a population-level have therefore the clear potential to provide real-time data for the assessment of community-wide health. Such biosensors can potentially be used in sewage matrices as community sensors to assess urinary and fecal biomarkers/pathogens for the monitoring of public health using WBE, while also providing a means of collecting data for epidemiological and socio-economic studies or as early-warning systems.

Publication 3. A new label-free electrochemical DNA (E-DNA) biosensor using a custom synthesized ferrocenyl (Fc) double-stranded DNA (dsDNA) intercalator as a redox marker is presented. Single-stranded DNA (ssDNA) was co-immobilized on gold electrodes with 6-mecarpto-hexanol (MCH) to control the surface density of the ssDNA probe, and hybridized with complementary DNA. The binding of the Fc intercalator to dsDNA was measured by differential pulse voltammetry (DPV). This new biosensor was optimized to allow the detection of single base pair mismatched sequences and, able to detect as low as 10 pM target ssDNA with a dynamic range from 10 pM to 100 nM. DNA extracted from wastewater was analysed by quantitative polymerase chain reaction (qPCR) targeting human-specific mitochondrial DNA (mtDNA). The aim of this approach is to enable the analysis of population biomarkers in wastewater for the evaluation of public health using WBE. The E-DNA biosensor was employed to detect human-specific mtDNA from wastewater before and after PCR amplification. The results demonstrate the feasibility of detecting DNA biomarkers in wastewater, which may allow the further development of DNA population biomarkers for public health using WBE.

Publication 4. A novel strategy for DNA aptamer immobilization was developed for the sensitive

electrochemical detection of a protein biomarker, with prostate specific antigen (PSA) as a case biomarker. Thiolated single-stranded DNA (ssDNA) was co-immobilized with 3-mercapto-1-propanol on gold electrodes, and used as a scaffold for DNA aptamer attachment through hybridization of the aptamer overhang (so-called "DNA-directed immobilization aptamer sensors", DDIAS). In the approach, the complementary DNA aptamer against PSA was assembled by the probe ssDNA onto the electrode to detect PSA; or the probe ssDNA directly hybridized with a complementary DNA aptamer/PSA complex following their pre-incubation in solution, so-called 'on-chip' and 'in-solution' methods, respectively. A double stranded DNA intercalator with a ferrocenyl (Fc) redox marker was synthesized to evaluate the feasibility of the strategy. The results demonstrated that the 'in-solution' method offers a favourable medium (in a homogeneous solution) for the binding between the aptamer and PSA, which shows to be more efficient than the 'on-chip' approach. DDIAS showed promising analytical performance under optimized conditions with a limit of detection in the range of fM and low non-specific adsorption.

Publications

1. Yang Z, Castrignanò E, Estrela P, Frost CG, Kasprzyk-Hordern, B. Community Sewage Sensors towards Evaluation of Drug Use Trends: Detection of Cocaine in Wastewater with DNA-Directed Immobilization Aptamer Sensors. **Sci Rep**, 2016 (6), 21024.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4753446/pdf/srep21024.pdf

2. Yang Z, Kasprzyk-Hordern, B, Frost CG, Estrela P, Thomas KV. Community Sewage Sensors for Monitoring Public Health. ***Environ Sci Technol*** 2015. 49 (10), 5845–5846.

http://pubs.acs.org/doi/abs/10.1021/acs.est.5b01434

3. **Yang Z**, Anglès d'Auriac M, Kasprzyk-Hordern B, Thomas KV, Frost CG, Estrela P. A novel DNA biosensor using a ferrocenyl intercalator applied to the potential detection of human population biomarkers in wastewater ***Environ Sci Technol*** 2015, 49 (9), 5609–5617. (IF 5.3)

http://pubs.acs.org/doi/abs/10.1021/acs.est.5b00637

4. **Yang Z**, Kasprzyk-Hordern, B, Goggins S, Frost CG, Estrela P. A novel immobilization strategy for electrochemical detection of cancer biomarkers in sewage: DNA-directed immobilization of aptamer for sensitive detection of prostate specific antigen. ***Analyst*** 2015, 140, 2628-2633. (IF 4.1)

http://pubs.rsc.org/en/Content/ArticleLanding/2015/AN/C4AN02277G#!divAbstract

Conference

1. "Community Sewage Sensors for Evaluation of Illicit Drug Use Trends by Wastewater-Based Epidemiology", The World Congress on Biosensors - Biosensors 2016, Gothenburg, Sweden, May 27-29, 2016.
2. "Community Sensors for Monitoring of Public Health by Means of Wastewater-Based Epidemiology", Testing the Waters Conferences 2015, Monte Verità, Ascona, Switzerland, October 11-15, 2015.
3. "Electrochemical Community Sensors for Monitoring of Public Health at Population Level Using Wastewater-Based Epidemiology", The Fifteenth International Symposium on Electroanalytical Chemistry (15th ISEAC), Changchun, China, August 13-16, 2015.
4. RSC Bioanalytical Sensing Technologies, London, UK, June 16, 2015 (Poster)
5. 4th International Conference on Bio-Sensing Technology, Lisbon, Portugal. May 10-13, 2015 (Poster)
6. Sensor in Medicine, London, UK, March 25-26, 2014. (Poster)

D2.4 Report on the development of analytical tools for the identification, confirmation and quantification of new psychoactive substances and their transformation products in sewage

An important part of WP2 was dedicated to the development and validation of LC/MS based analytical methods for new illicit drugs/psychoactive compounds and their metabolites in sewage. Their implementation aimed at gathering information regarding current market trends for illicit drugs. By using high resolution MS techniques (Orbitrap and QTOF), the retrospective analysis of sewage extracts may help in establishing time trends of new compounds and their metabolites. As a complementary tool to the European Early-Warning System (EEWS), which provides information about new psychoactive substances (NPS) on the market, WBE can complement objectively the monitoring of illicit (new) drugs, because results can be obtained in a short time frame, being thus ideal for detecting changes in the increasingly dynamic and fast-moving nature of the illicit drugs problem.

The application of (Q)TOF MS for detection and identification of organic compounds in surface water and sewage water was investigated by the three ESRs (**ESR7, ESR8, and ESR9**), together with the development of advanced methods for rapid screening of a wide range of compounds, presenting quite distinct polarities, with minimal sample manipulation. The excellent capabilities of TOF analyzers (full spectrum acquisition, high sensitivity, and accurate mass measurements) together with the complementary use of GC and LC enables the identification of much higher number of compounds in water than with most analytical approaches.

Degradation experiments were performed in sewage and surface water using LC-QTOFMS, which is more appropriate taking into account the higher polarity of the transformation products (TPs). MS and MS/MS experiments have been used in the identification and elucidation of TPs that can be relevant for monitoring water quality. In a final step, the work focused on the quantification of drugs, metabolites and TPs identified in laboratory degradation experiments and those detected in a preliminary screening in order to widen the monitoring applied at present and to obtain more realistic data. Multi-class quantitative methods based on LCMS/MS with QqQ would need to be modified or newly developed.

Publication 1. An analytical method using liquid chromatography coupled to positive electrospray tandem mass spectrometry (LC-ESI-MS/MS) was validated for the determination of seven NPS in sewage: methoxetamine (MXE), butylone, ethylone, methylone, methiopropamine (MPA), 4-methoxy-methamphetamine (PMMA), and 4-methoxyamphetamine (PMA). Sample preparation was performed using solid-phase extraction (SPE) with Oasis MCX cartridges. The LC separation was done with a HILIC (150 x 3 mm, 5 µm) column which ensured good resolution of the analytes with a total run time of 19 min. The lower limit of quantification (LLOQ) was between 0.5 and 5 ng/L for all compounds. The method was validated by evaluating the following parameters: sensitivity, selectivity, linearity, accuracy, precision, recoveries and matrix effects. The method was applied on sewage samples collected from sewage treatment plants in Belgium and Switzerland in which all investigated compounds were detected, except MPA and PMA. Furthermore, a consistent presence of MXE has been observed in most of the sewage samples at levels higher than LLOQ.

Publication 2. Accurate-mass mass spectrometry (AMMS) using a quadrupole time-of-flight (QTOF) analyzer can be useful for wide-scope screening since it provides sensitive, full-spectrum MS data. We have developed a qualitative screening workflow based on data-independent acquisition mode (all-ions MS/MS) on liquid chromatography (LC) coupled to QTOFMS for the detection and identification of NPS in biological matrices. The workflow combines and structures fundamentals of target and suspect screening data processing techniques in a structured algorithm. This allows the detection and tentative identification of NPS and their metabolites. We have applied the workflow to two actual case studies involving drug intoxications where we detected and confirmed the parent compounds ketamine, 25B-NBOMe, 25C-NBOMe, and several predicted phase I and II metabolites not previously reported in urine and serum samples. The screening workflow demonstrates the added value for the detection and identification of NPS in biological matrices.

Publication 3. Phenethylamine-based designer drugs are prevalent within the NPS market. Characterisation of their metabolites is important in order to identify suitable biomarkers which can be used for better monitoring their consumption. The objective of this study was to stepwise investigate the in vitro human metabolism of seven phenethylamine-based designer drugs using individual families of enzymes. The following NPS were included: para-methoxyamphetamine, para-methoxymethamphetamine, 4-methylthioamphetamine, N-methyl-benzodioxolylbutanamine, benzo-dioxolylbutanamine, 5-(2-aminopropyl) benzofuran, and 6-(2-aminopropyl) benzofuran. Identification and structural elucidation of the metabolites was performed using LC-QTOFMS. The targeted drugs were mainly metabolised by cytochrome P450 enzymes via O-dealkylation as the major pathway, followed by N-dealkylation, oxidation of unsubstituted C atoms and deamination (to a small extent). These drugs were largely free from Phase II metabolism. Only a limited number of metabolites were found which was consistent with the existing literature for other phenethylamine-based drugs. Also, the metabolism of most of the targeted drugs progressed at slow rate. The reproducibility of the identified metabolites was assessed through examining formation patterns using different incubation times, substrate and enzyme concentrations. Completion of the work has led to a set of metabolites which are representative for specific detection of these drugs in intoxicated individuals and also for meaningful evaluation of their use in communities by WBE.

Publication 4. A qualitative screening of NPS was carried out in 27 pooled urine samples collected from a city center in the UK and festivals in the UK and Belgium. The analysis method was based on data-independent acquisition mode (all-ions MS/MS) using LC-QTOFMS. An in-house library was used with more than 1500 entries corresponding to NPS, classical drugs and metabolites. All samples contained 56 and 28 compounds of interest from the UK and Belgium, respectively. Of the different compounds detected, 35% were confirmed using reference standards and the remaining compounds were tentatively identified using elucidated fragmentation pathways. The highest numbers of NPS identified in both countries were from the cathinone and phenylethylamine families, with a higher number being detected in samples from the festival in the UK. Several cathinone metabolites in human urine were detected and identified. The screening method proved useful to detect a large number of compounds and to determine the frequently used NPS.

Publication 5. A simple, fast and reliable analytical method for the determination of phosphodiesterase type V inhibitors in wastewater was developed and validated. The method was based on direct injection followed by liquid chromatography coupled to tandem mass spectrometry with triple quadrupole as mass analyzer. Transformation products and analogues were included in the target list besides the three active pharmaceutical ingredients (sildenafil, vardenafil, and tadalafil). The method performance was thoroughly investigated, including the analyte stability in wastewater and matrix effect. All target compounds presented linear fits between their LOD and 500 ng/L. The quantification limits ranged from 1.6 to 30 ng/L for all compounds except for n-octylnortadalafil (LOQ: 100 ng/L); precision calculated as intraday repeatability was lower than 30%; accuracy calculated as procedural recovery ranged successfully between 85 and 105% in all cases. The method was applied to samples collected during three week-long monitoring campaigns performed in 2013, 2014 and 2015 in three Dutch cities. Only sildenafil and its two metabolites, desmethyl- and desethylsildenafil, were present with normalized loads ranging from LOQ to 8.3, 11.8 and 21.6 mg/day/1000 inh, respectively. Two additional week-long sets of samples were collected in Amsterdam at the time that a festival event took place, bringing around 350,000 visitors to the city. The difference in drug usage patterns was statistically studied: "weekday" versus "weekend", "normal" versus "atypical" week; and results discussed. The metabolite to parent drug concentration ratio evolution during consecutive years was discussed, leading to several possible explanations that should be further investigated. Finally, the WBE approach was applied to back-calculate sildenafil consumption.

Publication 6. A screening approach was applied to influent and effluent wastewater samples. After injection in a LC-LTQ-Orbitrap, data analysis was performed using two deconvolution tools, MsXelerator (modules MPeaks and MS Compare) and Sieve 2.1. The outputs were searched incorporating an in-house database of >200 pharmaceuticals and illicit drugs or ChemSpider. This hidden target screening approach led to the detection of numerous compounds, including the illicit drug cocaine and its metabolite benzoylecgonine and the pharmaceuticals carbamazepine,

gemfibrozil, and losartan. The compounds found using both approaches were combined, and isotopic pattern and retention time prediction were used to filter out false positives. The remaining potential positives were re-analysed in MS/MS mode and their product ions were compared with literature and/or mass spectral libraries. The inclusion of the chemical database ChemSpider led to the tentative identification of several metabolites, including paraxanthine, theobromine, theophylline and carboxylosartan, as well as phenazone. The first three of these compounds are isomers and they were subsequently distinguished based on their product ions and predicted retention times. The use of deconvolution tools facilitates non-target screening and enables the identification of a higher number of compounds.

Publication 7. Using HRMS, compound identification relies on the high mass resolving power and mass accuracy attainable by these analyzers. When dealing with wide-scope screening, retention time prediction can be a complementary tool for the identification of compounds, and can also reduce tedious data processing when several peaks appear in the extracted ion chromatograms. There are many in silico, Quantitative Structure-Retention Relationship methods available for the prediction of retention time for LC. However, most of these methods use commercial software to predict retention time based on various molecular descriptors. This paper explores the applicability and makes a critical discussion on a far simpler and cheaper approach to predict retention times by using LogKow. The predictor was based on a database of 595 compounds, their respective LogKow values and a chromatographic run time of 18 min. Approximately 95% of the compounds were found within 4.0 min of their actual retention times, and 70% within 2.0 min. A predictor based purely on pesticides was also made, enabling 80% of these compounds to be found within 2.0 min of their actual retention times. To demonstrate the utility of the predictors, they were successfully used as an additional tool in the identification of 30 commonly found emerging contaminants in water. Furthermore, a comparison was made by using different mass extraction windows to minimize the number of false positives obtained.

Publication 8. The present work investigates the use of the most popular HRMS instruments, QTOF and linear trap quadrupole-Orbitrap, from two different laboratories. A suspect screening for PIDs was carried out on wastewater (influent and effluent) and surface water samples from Castellón, Eastern Spain, and Cremona, Northern Italy, incorporating a database of 107 PIDs (including 220 fragment ions). A comparison between the findings of both instruments and of the samples was made which highlights the advantages and drawbacks of the strategies applied in each case. In total, 28 compounds were detected and/or identified by either/both instruments with irbesartan, valsartan, benzoylecgonine and caffeine being the most commonly found compounds across all samples

Publication 9. A large screening of around 1,000 emerging contaminants, focused on licit and illicit drugs and their metabolites, has been made in urban wastewaters and surface waters from the area of Bogotá, Colombia. After a simple generic solid-phase extraction (SPE) step with Oasis hydrophilic-lipophilic balanced (HLB) cartridges, analyses were made by UHPLC-QTOF MS under MS(E) mode (sequential acquisition of mass spectra at low energy (LE) and high collision energy (HE)). Accurate mass measurements and the information provided by MS(E) on the presence of the (de)protonated molecule and fragment ions allowed the reliable identification of the compounds detected, even without reference standards being available in some cases (tentative identification). The compounds most frequently found were acetaminophen/paracetamol, carbamazepine and its dihydro-dihydroxylated metabolite, clarithromycin, diclofenac, ibuprofen, gemfibrozil, lincomycin, losartan, valsartan, the two metabolites of metamizole (4-acetamido-antipyrine and 4-formylamino-antipyrine), sucralose, and cocaine and its main metabolite benzoylecgonine. Caffeine, the sweetener saccharin, and two hydroxylated metabolites of losartan were tentatively identified in almost all samples analyzed. Pharmaceutical lidocaine was tentatively identified and subsequently confirmed with reference standard. For the first time, a general overview of the occurrence of drugs and their metabolites in the aquatic environment of Colombia has been reported.

Publication 10. The recent development of broad-scope high resolution mass spectrometry (HRMS) screening methods has resulted in a much improved capability for new compound identification in environmental samples. However, positive identifications at the ng/L concentration level rely on analytical reference standards for chromatographic retention time (tR) and mass spectral comparisons. Chromatographic tR prediction can play a role in increasing confidence in suspect screening efforts for new compounds in the environment, especially when standards are not available, but reliable methods are lacking. This work focuses on the development of artificial neural networks (ANNs) for tR prediction in gradient RPLC and applied along with HRMS data to suspect screening of wastewater and environmental surface water samples. Based on a compound tR dataset of >500 compounds, an optimized 4-layer back-propagation multi-layer perceptron model enabled predictions for 85% of all compounds to within 2 min of their measured tR for training (n=344) and verification (n=100) datasets. To evaluate the ANN ability for generalization to new data, the model was further tested using 100 randomly selected compounds and revealed 95% prediction accuracy within the 2-minute elution interval. Given the increasing concern on the presence of drug metabolites and other transformation products (TPs) in the aquatic environment, the model was applied along with HRMS data for preliminary identification of pharmaceutically-related compounds in real samples. Examples of compounds where reference standards were subsequently acquired and later confirmed are also presented. To our knowledge, this work presents for the first time, the successful application of an accurate retention time predictor and HRMS data-mining using the largest number of compounds to preliminarily identify new or emerging contaminants in wastewater and surface waters.

Publications

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Conferences

1. 53rd TIAFT -The International Association of Forensic Toxicology meeting 2015 Florence – August 30th - September 4th 2015. "*Detection and identification of new psychoactive substances in pooled urine using liquid chromatography coupled to high resolution mass spectrometry*." (**Kinyua J -** Presenting Author)

2. ChemCYS Blakenberge, Belgium 27-28 February 2014. "*Development of a new method for the analysis of 'amphetamine like' new psychoactive substances in wastewater*. " (**Kinyua J -** Presenting Author)

3. 2nd International conference on Wastewater-based drug epidemiology -Testing the Waters II Monte Verità, Ascona, Switzerland,11-15 October 2015. "*Illicit drugs and alcohol use among different groups of population in Lesvos Island.*" (**Kinyua J -** Presenting Author)

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Antwerpen, 09/07/2016

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