

This is an Accepted Manuscript of the following article:

J.A. Baz-Lomba, Malcolm J. Reid, Kevin V. Thomas. Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF. *Analytica Chimica Acta*.

Volume 914, 2016, pages 81-90, ISSN 0003-2670.

The article has been published in final form by Elsevier at

<http://dx.doi.org/10.1016/j.aca.2016.01.056>

© 2016. This manuscript version is made available under the

CC-BY-NC-ND 4.0 license

<http://creativecommons.org/licenses/by-nc-nd/4.0/>

It is recommended to use the published version for citation.

1 Target and suspect screening of psychoactive substances in sewage-based samples 2 by UHPLC-QTOF

3 J.A. Baz-Lomba^{a,b}, Malcolm J. Reid^a, Kevin V. Thomas^a

4 ^aNorwegian Institute for Water Research, Gaustadalléen 21, NO-0349 Oslo, Norway

5 ^bFaculty of Medicine, University of Oslo, PO box 1078 Blindern, 0316 Oslo, Norway

6 Abstract

7 The quantification of illicit drug and pharmaceutical residues in sewage has been shown to be a valuable tool
8 that complements existing approaches in monitoring the patterns and trends of drug use. The present work
9 delineates the development of a novel analytical tool and dynamic workflow for the analysis of a wide range of
10 substances in sewage-based samples. The validated method can simultaneously quantify 51 target psychoactive
11 substances and pharmaceuticals in sewage-based samples using an off-line automated solid phase extraction
12 (SPE-DEX) method, using Oasis HLB disks, followed by ultra-high performance liquid chromatography
13 coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOF) in MS^e. Quantification and matrix
14 effect corrections were overcome with the use of 25 isotopic labeled internal standards (ILIS). Recoveries were
15 generally greater than 60% and the limits of quantification were in the low nanogram-per-liter range (0.4-187
16 ng L⁻¹). The emergence of new psychoactive substances (NPS) on the drug scene poses a specific analytical
17 challenge since their market is highly dynamic with new compounds continuously entering the market. Suspect
18 screening using high-resolution mass spectrometry (HRMS) simultaneously allowed the unequivocal
19 identification of NPS based on a mass accuracy criteria of 5 ppm (of the molecular ion and at least two
20 fragments) and retention time (2.5 % tolerance) using the UNIFI screening platform. Applying MS^e data
21 against a suspect screening database of over 1,000 drugs and metabolites, this method becomes a broad and
22 reliable tool to detect and confirm NPS occurrence. This was demonstrated through the HRMS analysis of three
23 different sewage-based sample types; influent wastewater, passive sampler extracts and pooled urine samples
24 resulting in the concurrent quantification of known psychoactive substances and the identification of NPS and
25 pharmaceuticals.

26 Keywords

27 High resolution mass spectrometry, Sewage-base epidemiology, Multi-residue method, Dynamic workflow,
28 Screening, Psychoactive drugs

29 **1. Introduction**

30 The illicit use of psychoactive substances is a worldwide phenomenon with incalculable consequences on
31 society. Estimates from 2012, indicate that between the 4 and 7 % of the world's population has used an illicit
32 substance at least once during the previous year [1]. Even though the overall European drug situation is
33 generally stable, a graduated and complex situation is emerging [2]. Classic drugs, such as heroin, cocaine and
34 cannabis remain dominant but the drug market is now peppered with a vast array of synthetic psychoactive
35 substances. The EU Early Warning System for first time notified the discovery of 101 new psychoactive
36 substances (NPS) in 2014 [3]. Illicit drug use and prevalence estimations are typically based on population
37 surveys, clinical cases, seizures and mortality rates related to use. In addition to known problems with bias and
38 a lack of representativeness, these approaches also require a long time to establish a general overview of the
39 illicit drug situation and, generally, results are published several years post survey [4].

40 Pharmaceuticals arising from human, veterinary and aquaculture use, occur in the aquatic environment as one
41 of the largest sources of contaminants of anthropogenic origin and have recently become a new environmental
42 problem [5]. Many studies have been performed assessing the impact of hospitals and their contribution to the
43 pharmaceutical loads found in wastewater [6-8], the efficiency of the removal of pharmaceuticals by
44 wastewater treatment plants (WWTP) [9], the spatial and temporal differences of pharmaceuticals occurrence
45 in wastewater [10] and recently to compare the results of the wastewater analysis with predicted concentrations
46 based on prescription figures [11, 12]. In the context of sewage-based epidemiology (SBE) pharmaceutical
47 residues also offer the potential to map certain types of disease and other health based stressors[13].

48 The measurement of illicit drugs and pharmaceuticals in wastewater has become an extended topic over the
49 past decade and SBE has been established as a complementary approach for monitoring trends of illegal drug
50 use [14-18]. Recently the approach has been evaluated with an international study comparing the drug
51 consumption in 21 different countries by quantitatively analyzing illicit drugs and their metabolites in 47
52 WWTP [19, 20]. SBE has been used to estimate drug use in communities (i.e. city, small town, school, prisons)
53 as well as identify changes in drug consumption during special events [21, 22], temporal changes [17], and
54 providing complementary data on the use of NPS [23-25]. Thus far, the analytical methods used for SBE have
55 focused on the principal challenges related to the low concentrations of drug residues in wastewater combined
56 with the complexity of the wastewater matrix. These procedures have been mainly developed for classic illicit
57 drugs (cocaine, amphetamines, opioids and cannabis) and a broad range of pharmaceuticals, and based on
58 solid-phase extraction (SPE) for sample pre-treatment and pre-concentration followed by instrumental analysis,
59 typically liquid chromatography coupled to tandem mass spectrometry (QqQ)[5, 16].

60 The hyphenation of liquid chromatography to high resolution mass spectrometry (HRMS), i.e. orbitrap and
61 quadrupole time-of-flight (QTOF), has increased the potential of SBE to identify NPS and their residues in
62 sewage based samples. HRMS offers some unique advantages for screening and identification, such as better
63 specificity due to its increased resolving power and the possibility to perform retrospective analyses without the
64 need of additional sample analyses [26].

65 Since the NPS market is in constant flux, new strategies are urgently required. New analytical methods based
66 on QqQ for the target analysis of NPS are continuously being published[24, 25] but in general these techniques
67 cannot keep up with the rapidly evolving NPS market making necessary the development of new approaches
68 that combine target analysis with wide-scope suspect screening capable of confirming the presence/absence of
69 a new compounds with quantification should reference standards be available[27]. This challenge can be
70 overcome by using a suspect screening approach that has already been demonstrated as a suitable technique for
71 the detection and confirmation of several organic compounds[27], including NPS[23, 28]. The simultaneous
72 determination of a broad number of compounds in one injection, with a corresponding reduction of time and
73 costs, without the need for reference standards make this approach one of the current trends in environmental
74 analytical chemistry.

75 The aim of the presented work was to establish a broad analytical tool for the analysis of different types of
76 drugs in sewage-based samples, such as pooled urine, wastewater and passive sampler extracts and develop a
77 dynamic workflow for the introduction of new substances of interest in the future due to the high potential of
78 the HR-MS instruments for the identification of suspect compounds presented in the sample.. Firstly a targeted
79 multi-residue method for the simultaneous identification and quantification of 51 drugs, pharmaceuticals and
80 their main urinary metabolites by UHPLC/QTOF was validated as recommended by EU guidelines with minor
81 modifications [29, 30]. With the target method established to track the quantitative occurrence of selected
82 target analytes, the secondary goal of this work was to detect possible NPS and other compound of possible
83 interest in sewage-based samples thought the use of a suspect screening approach. The present work also
84 describes the potential of non-target analysis for identifying NPS and their subsequent inclusion into the
85 suspect database or target method if standard reference is available through a dynamic approach.

86 **2. Materials and methods**

87 Detailed information relating to reagents and chemicals (illicit drugs, pharmaceuticals and metabolite reference
88 substances), the sewage-based samples and extraction procedure can be found in the Supplementary
89 Information (SI).

90

91 2.1 Sewage-base samples

92 Influent samples were collected from two Norwegian WWTPs; VEAS WWTP in Oslo and Ladehammeren and
93 Høyvingen WWTP in Trondheim. The robustness of the method was also examined by analysing different
94 sewage-based samples. The “Pharmaceuticals” version of the polar organic chemical integrative sampler
95 (POCIS) was placed for two weeks in the sedimentation overflow channel at the Oslo WWTP and the pooled
96 urine samples were collected from three different Norwegian music festivals. **Figure 1** shows an overview of
97 the samples used in this study (detailed information provided in **SI**).

98 2.2 Extraction procedure

99 Defrosted sewage samples (100 mL) were spiked with a mix of 25 isotope labeled internal standards (ILIS) to
100 give a concentration of 400 ng L⁻¹. The pH was controlled but generally was the desired (~7). Subsequently,
101 extraction was performed using a SPE-DEX fully automatable extraction system (Horizon Technology, Salem,
102 NH, USA) using HLB (Hydrophilic-Lipophilic Balance) extraction disks (47 mm, I.D.; Horizon Technology,
103 City, Country) (more information in **SI** and **Table S-1**).

104 The selection of the Oasis HLB extraction sorbent was based on previous experience. Oasis HLB and MCX
105 generally yield the highest recoveries for most of the drugs and pharmaceuticals [5, 18]. Compared with Oasis
106 MCX, HLB provides a lower selectivity for some basic compounds, such as amphetamine-like compounds and
107 a considerable increase of the matrix components retained in the sorbent leading to a higher sensitivity. Despite
108 this, HLB, with a mixed-mode cation exchange sorbent, offers the possibility to extract a wide range of
109 compounds with different psychochemical characteristics suiting the goal of the method, enabling the
110 simultaneous analysis of the widest range of drugs and pharmaceuticals in one single extraction.

111 To determine the method recovery influent wastewater from VEAS WWTP in Oslo was spiked in quintuplicate
112 with a standard mixture containing all of the target analytes at two different concentrations together with a
113 mixture of the 25 ILIS. The two concentrations were selected according to those generally found in wastewater
114 influent.

115 2.3 Ultra-high pressure liquid chromatography

116 The chromatography column, stationary phase and mobile phases were selected in accordance with a pre-
117 established screening method[31]. **Figure 2** shows the 2D mass chromatogram of the low energy channel (6eV
118 ESI⁺) for the optimal separation of all target compounds and NPS at a concentration of 250 ng mL⁻¹. A Waters
119 Acquity UPLC system (Milford, MA, USA) was used for this work. Chromatographic separation was carried
120 out using an Acquity UPLC HSS C18 column (2.1 x 150 mm, particle size 1.8 µm) (Waters, Milford, MA,
121 USA). Gradient elution was performed at a constant flow of 0.4 ml min⁻¹ using 5 mM ammonium formate, pH

122 3.0 (solvent A) and acetonitrile with 0.1 % formic acid (solvent B). The gradient elution starts with 87% A and
123 then increasing B to 95% in 15 minutes: Solvent A, held for 0.5 min; 0.5-10 linear rate to 50% B, 10-10.75
124 linear rate to 95% B, held for 0.5 min; reconditioning with a linear rate to 87% A, 12.50-15 min. The analytical
125 column and the guard column were kept at 50 °C and the sampler manager at 5 °C. The weak and strong wash
126 used to remove the contaminants from the needle and the injection port were 10% ACN in water (600 µL) and
127 95% ACN in water (200 µL) respectively.

128 2.4 Quadrupole Time-of-flight Mass Spectrometry

129 A Xevo G2-S Q-TOF mass spectrometer (Waters, Milford, MA USA) was used in positive ESI mode for
130 acquisition using MS^c, that allows both precursor and product ion data to be simultaneously acquired during a
131 single run. The MS method consists of 3 functions, the first (low energy, LE) applies collision energy of 6 eV,
132 the second function (high energy, HE) acquires through a collision energy ramp of 15-50 eV and the third
133 function acquires the lock mass data for online mass calibration. The MS range is 70-700 with a scan time of
134 0.1 second in continuum mode, preserving the peak shape of the exact-mass precursor and product ions. The
135 source conditions whose maximum intensities were achieved were the following: capillary voltage 3 kV,
136 sample cone, 20 V, source offset 80 V, source temperature 120 °C, desolvation temperature 500 °C, cone gas
137 flow rate 50 L h⁻¹, desolvation gas (N₂) flow rate 1000 L h⁻¹.

138 The mass spectrometer was calibrated using a solution of sodium formate over a mass range of 50-1000 Da.
139 Analyses were performed using an external reference (Lock-Spray™). During the data acquisition the mass
140 was corrected using an external reference (Lock-Spray™) consisting of 0.2 µg mL⁻¹ solution of leucine-
141 enkephalin infused continuously at 10 µL min⁻¹ via a lockspray interface. The lock mass data were acquired
142 every 20 seconds for 0.1 seconds and for the rest of the time the baffle in the ion source blocked the entry of
143 the lock spray. This generated a reference ion in positive mode at *m/z* 556.2771 that was used for real-time mass
144 corrections in order to maintain the mass accuracy and reproducibility.

145 2.5 Data processing

146 The workflow and identification confidence used in this work were based upon those described by Krauss et al.
147 [32] and Schymanski et al. [33](**Figure 3**).

148 The MSe data processing using the UNIFI screening platform (Waters Corporation, Milford MA, USA) was
149 performed in two steps. Firstly, all the continuum data was peak detected using a 3D peak algorithm based on
150 the calculation of the peak volumes by the detection of all the ion crests in a given mountain range. This
151 provides a complete list of retention-time / *m/z*-pairs which are then used for screening. The second step which
152 follows peak detection is the screening protocol where Unifi matches the observed *m/z* retention-time pairs

153 against the scientific database using defined settings such as the retention time accuracy (2.5 %), mass accuracy
154 of parent ion and fragment ions (5 ppm) and theoretical isotope patterns for the protonated molecule at LE and
155 at least two accurate fragment ions at HE [30]. The database is built from a pre-established screening method
156 [31] which uses the same chromatographic and detector parameters as in this study.

157 For target screening the procedure is unchanged, except the screening is focused only on the 51 target
158 compounds (as opposed to the entire 1000 + library) and reference standards are run with the analysis to prove
159 quantitative information/results (**Table 1**). Protonated precursor ion data at LE was extracted and used for
160 quantification. For this work, selection of the 51 target analytes was based on their reported use in Norway and
161 their previously reported occurrence in wastewater [17, 19, 24].

162 For suspect screening, the retention-time/ m/z-pairs are compared against a broad list of approximately 1000
163 compounds (note that this database is under constant renewal and expansion so the exact number of compounds
164 varies over time). UNIFI also automatically assesses the candidate values with the fragments, mass defect,
165 isotope pattern and adducts (M+H, M+Na, M+NH₄). The suspect screening database (including mass spectra,
166 assigned fragments and retention time) was applied under the same acquisition parameters as above and tested
167 in this work on pooled urine samples in an attempt to identify NPS or possible compounds of interest to include
168 in the future in the target method through a reiterative development workflow.

169 While the current study used a screening database developed around a commercially product, and manually
170 augmented with 16 NPS, suspect screening may also be performed using other public databases (e.g.
171 MassBank, Chemspider) or in-silico fragmentation tools (MetFrag) which are available for many
172 environmental contaminants. Suspect screening lists may also be produced with computational prediction
173 systems that can provide a list of potential excreted biomarkers and biotransformation products in wastewater
174 [34].

175 Directly infusing the standards and subsequently analyzing the spectra usually calculate fragmentation
176 pathways. This study attempts to complement the suspect-screening database by importing suspect candidates
177 relying on accurate spectral information and different non-target tools. Where analytical data on fragmentation
178 is lacking the UNIFI fragment match tool applies a series of novel algorithms based on systematic bond
179 disconnections of the precursor structure to predict fragmentation pathways that can then be compared with
180 measured spectra in order to help to increase the amount and the reliability of the information essential for the
181 subsequent identification and confirmation of the compound of interest. This approach has been tested in this
182 study with two NPS. The common fragment approach is another interesting option for the detection of NPS.
183 Synthetic cannabinoids generally appear on the market with minor structural modifications which means that

184 during fragmentation they lose the same specific functional groups and that structural information can be used
185 as a filter to detect those components. This tool has been tested with 14 different synthetic cannabinoids.

186 2.6 Quantification and method validation

187 The performance of the method was evaluated following EU guidelines with minor modifications[29, 30]. The
188 instrumental linearity of the method was studied by analyzing standard solution in triplicate at 9 different
189 concentrations that were between 0.25 ng mL⁻¹ and 500 ng mL⁻¹ (it would be equivalent to 1-2000 ng L⁻¹ in
190 sewage after applying the pre-concentration factor). Satisfactory linearity was assumed when the correlation
191 factor (R²) was > 0.99, based on analyte/internal standard peak area ratio applied in different concentration
192 ranges for each compound. The most relevant aspects about the instrumental parameters are summarized in
193 **Table S-2.**

194 The theoretical limit of quantification (LOQ_{THE}) for real samples was calculated based on the ratio between the
195 instrumental limit of quantification, estimated as a signal-to-noise (S/N) ratio of 10 for the standard in the
196 calibration curve at the lowest level, and the matrix suppression (MS) for each deuterated internal standard
197 multiplied by the concentration factor (CF).

$$198 \quad \textit{Theoretical LOQ}_{RS} = \frac{LOQ_{inst}}{MS} \times CF$$

199 The matrix effect defined as Matuszewski et al.[35] was calculated as the ratio between the accurate mass
200 signal of the 25 deuterated standard spiked in different sewage samples after extraction and the accurate mass
201 signal of the same deuterated standard in the calibration curve. The influence of the sewage matrix
202 concentration on the analysis is a crucial factor but simply controlled with a pre-concentration factor study[36].
203 For analytes where deuterated standards were unavailable, either the deuterated analyte with similar structure,
204 polarity or the closest retention time was selected for correction.

205 Precision was expressed as the repeatability of the method in terms of relative standard deviation and trueness
206 was tested by means of the absolute recovery due to the lack of quality control standards. The relative
207 recoveries and standard deviations of the whole procedure were calculated by analyzing sewage water samples
208 spiked at a low and high concentration with the analytes (100 and 600 ng L⁻¹) according to the levels generally
209 found in wastewater and with 600 ng L⁻¹ of the deuterated internal standard solution mix. All recovery
210 experiments were performed in six replicates (n=6). Concentrations of target compounds found in wastewater
211 “blank” samples were subtracted from the spiked samples due to the absence of an appropriate wastewater
212 blank. Instrumental accuracy and precision was assessed by analyzing spiked Mili-Q water (n=5) at two

213 different concentrations for each compound, low level (between points 2-3 on the calibration curve) and high
214 level (between points 8-9 on the calibration curve).

215 The stability of the target analytes in wastewater was evaluated in triplicate (n=3) via the analysis of 500 mL of
216 spiked (1000 ng mL⁻¹) wastewater immediately following spiking (t=0 hours) and again at t=5, 8 and 120 hours
217 post spiking. The wastewater sample (pH 7.5) was stored in an amber glass bottle at 10 °C throughout the study
218 in order to best replicate the average wastewater influent temperature.

219

220 3. RESULTS AND DISCUSSION

221 3.1 Sample extraction optimization

222 Selected parameters were investigated in order to optimize the method. Silanisation of the glassware, by
223 deactivating the ion-exchange groups with dichlorodimethylsilane, resulted in increased recoveries for some of
224 the more polar drugs, such as amphetamine-like compounds (Figure S-1). Elevated evaporation temperature
225 was also found to result in analyte losses, therefore special attention was paid to the drying of extracts .Figure
226 S-2 shows poor recoveries for some of the phenethylamines following nitrogen evaporation at 50°C. All
227 samples were subsequently evaporated to around 100 µL under a gentle nitrogen stream at 35 °C.

228 3.2 Method performance

229 The relative recoveries and standard deviations from the relative responses analyte/ILIS are shown in the **Table**
230 **S-3**. For the target analytes with corresponding ILIS the recoveries are typically >80% with some exceptions
231 such as MDMA, norketamine and atomoxetine with slightly lower recoveries of around 70%. The remaining
232 target analytes were corrected using the ILIS with the closest structure, polarity or eluting time, achieving
233 relative recoveries ranging from 60 to 118%. However certain analytes such as clonazepam (56%),
234 dehydronorketamine (53%), methoxetamine (40%) and methylphenidate (50%) showed lower recoveries.
235 Relative recoveries at 100 ng L⁻¹ are in general slightly lower due their proximity to the LOQ_{THE}, however in
236 general the recoveries do not substantially differ between the low and high concentration. The overall method
237 precision was calculated as the relative standard deviation (RSD) due to the lack of reference materials for the
238 wastewater samples, and showed satisfactory results <20% RSD for all compounds.

239 Paracetamol and hydroxycotinine were detected at high concentrations in the blank (low ppb) making
240 subtraction and the recovery calculation not possible for paracetamol, and less precise for hydroxycotinine
241 (highest RSD). Carbamazepine also exhibits ionization issues in terms of signal enhancement with recoveries
242 slightly higher than 100% while other compounds with high signal suppression were gabapentin, *p*-

243 hydroxymethamphetamine, MDA, diclofenac, THC-OH and THC-COOH, which were finally excluded from
244 further validation in this study, but this does not however preclude the potential for lower matrix effects and
245 better analytical outcomes in wastewater from other locations or other sample types that present a cleaner
246 matrix.

247 The overall method recoveries were satisfactory and similar to those screening methods previously reported for
248 pharmaceuticals in wastewater samples [5]. Different target procedures, such as QqQ, with optimized and
249 specific conditions for each analyte, generally present greater sensitivity. This is a downside of all multi-
250 residue methods where the optimized conditions are compromised in order to accommodate several analytes,
251 especially in complex and variable matrix such as wastewater. Nevertheless the good sensitivity levels and
252 dynamic range presented in the new HR-MS instruments together with the instrumental performance in terms
253 of repeatability and sensitivity helps to counteract low recoveries.

254 3.3 Quantification and method validation

255 **Table S-2** provides an overview of the performance of the developed target method. The instrumental linearity
256 for nine concentration levels is in the range of 0.25 to 400 ng mL⁻¹, equivalent to 1-2,000 ng L⁻¹ in wastewater
257 after applying the pre-concentration factor. These are within the levels reported in literature[16] showing good
258 results for all of the initial 51 selected compounds presenting correlation coefficients greater than 0.99.
259 Different ranges were applied for every compound according to sensitivity. Instrumental LOQ was calculated
260 as the signal-to-noise (*S/N*) ratio of 10 for the standard in the calibration curve at the lowest concentration.
261 Instrumental accuracy and precision, both for intra- and inter-day, was assessed at two different concentration
262 levels for each compound showing an satisfactory accuracy levels between 87.8-113.1 % range and precision
263 <12.8 % (RSD).

264 The theoretical limits of quantification (LOQ_{THE}, **Table S-3**), calculated based on the ratio between the
265 instrumental limit of quantification and matrix suppression multiplied by the concentration factor for each
266 deuterated internal standard, varied between 0.4 and 187 ng L⁻¹. This is just a valuable estimation about the
267 method performance and due to the complexity of the matrix common sense has to be used prior the analysis of
268 a new batch of samples.

269 The instrumental performance was also evaluated in terms of the intra-day (repeatability) and inter-day
270 (reproducibility) precision studies from five spiked Mili-Q water samples at two different concentration levels
271 in five repeated injections and three consecutive days. The results in terms of RSD show values below 10% in
272 both intra and inter-day studies for all the compounds except for THC-COOH at the low concentration level for
273 the intra-day (10.2%) and amphetamine and flunitrazepam at the low concentration level for the inter-day (11.8

274 and 12.8 respectively). The good instrumental performance together with the high selectivity reached and low
275 mass error for all the target compounds (mass error range between -0.51 and 0.39 mDa, (**Table S-2**) ensure the
276 reliability of the instrument performance, an essential aspect in HRMS.

277 3.4 Liquid Chromatography-Mass Spectrometry

278 Suppression or enhancement of the target ion signal in LC-MS is a critical issue in wastewater analysis due to
279 the complex nature of the sample and the co-elution of the analytes with matrix constituents. The negative
280 consequences of this problem are best avoided with the use of appropriate ILIS, together with extensive sample
281 pre-treatment (clean-up), chromatographic separation and sample dilution. The effects of the sample matrix
282 was evaluated by comparing the matrix effect/pre-concentration factor ratio for all the 25 deuterated internal
283 standards. Deuterated internal standards are not present in sewage water samples but are affected by the same
284 potential losses during the analytical process as the analyte of interest. The influence of the SPE loading
285 volume on matrix suppression was optimized by spiking 100 ng of the deuterated internal standard mix
286 solution into the extracts of 3 different wastewater extracts at 4 different volumes (100, 200, 300, and 400 mL).
287 The accurate mass signal was compared with the accurate mass signal of the deuterated internal standards in
288 solvent. A loading volume of 100 mL was found to give the most satisfactory compromise for the analysis with
289 a pre-concentration factor of 250 (LC vial volume 400 μ L, injection volume 5 μ L) (**Figure S-3**).

290 Van Nuijs et al.[37]considered not including matrix effect studies in method validation, claiming that matrix
291 effects can be highly variable between samples. We agree on the fact that each individual sample has a
292 different composition, but we strongly recommend the matrix characterization of different batches of
293 wastewater samples when they come from different locations or affected by different conditions since an
294 appropriate sample dilution can improve substantially the general performance of the method. Generally
295 analytical methods rely on the validated LOQ but wastewater analysis required a more intense day-to-day
296 control.

297 3.5 Stability in wastewater

298 The stability of the analytes in wastewater was investigated to ensure the use of appropriate sample-storage and
299 handling procedures. The study was configured to estimate the degradation of our target compounds during the
300 in-pipe transit time for a single wastewater sample.

301 **Table S-4** shows the results for the stability test for the target analyte signal in non-filtered wastewater at pH
302 7.5 and 10°C expressed as the difference between initial and final concentrations at different time points ($t= 0$,
303 5, 8 and 120 hours). Time points were set according the mean residence time in the sewer system from Oslo to

304 the VEAS WWTP, 5 hours, the total treatment time in the plant, 3 hours, and finally a long exposure during
305 120 hours to provide an estimation of the biodegradation after 5 days.

306 Over the first 5 hours EDDP is the only analyte with major change in peak area (45% higher). Between 5 and 8
307 hours significant changes were also observed for hydroxycotinine, benzoylecgonine, methylphenidate the
308 concentration changed substantially ($>\pm 30\%$). The results following 120 hours show degradation for the many
309 of the target analytes, such as morphine (83%), *p*-hydroxymethamphetamine (70%), paracetamol (-88%), MDA
310 (-96%), methylphenidate (-88%), cocaine (-75%), EDDP (77%), nitrazepam (-79%) and phenazepam (-70%).

311 These data provide an indication of the transformation that may occur during in-sewer transport. The results are
312 in accordance with previous reports [38].

313 3.6 Application to sewage water samples

314 3.6.1 Target Screening.

315 The main objective of this work was to demonstrate the broad applicability of a multi-residue method in MS^e
316 by UHPLC-QTOF for both screening and quantification purposes. The validated method was applied to the
317 analysis of 15 wastewater samples collected from two WWTPs in Oslo and Trondheim in 2014 to assess the
318 applicability of the method. **Table 2** shows the population normalized loads for the target drugs in wastewater,
319 estimated from the daily concentration of each detected target compound in the wastewater sample, daily flow
320 discharged into the WWTP and by the population size ($\text{mg day}^{-1} 1000 \text{ inhabitants}^{-1}$). Results from Oslo show
321 the daily average loads calculated from the analysis in triplicate of a daily composite sample taken every
322 Friday, Saturday and Sunday during three consecutive weekends.

323 In general, pharmaceuticals were detected in higher concentration for both cities. Atenolol, paracetamol,
324 metoprolol, propranolol, citalopram, carbamazepine, oxazepam were detected at elevated concentrations in all
325 of the samples from Oslo and Trondheim. Methylphenidate and alprazolam were only present in samples from
326 Trondheim, suggesting different levels of consumption within the same country.

327 The heroin metabolites, 6-MAM and morphine, were detected in samples from both locations but it is
328 important to remember that while 6-MAM is specific to heroin, the presence of morphine in wastewater also
329 can result from the clinical use of legal pharmaceuticals containing morphine or codeine. Methadone and its
330 metabolite, EDDP, are usually present in wastewater samples and in this study the loads obtained in Oslo are
331 approximately ten times higher than those from Trondheim. Biomarkers of other commonly used drugs, such as
332 cocaine, benzoylecgonine, amphetamine and methamphetamine were detected in samples from both cities at
333 different concentrations. While the loads of cocaine/benzoylecgonine are approximately ten times higher in
334 Oslo than in Trondheim, the results for amphetamine and methamphetamine are slightly higher in Trondheim

335 suggesting different use patterns. This is in agreement with the general geographical distribution of the drugs in
336 Norway published in the Annual report to the European Monitoring Centre for Drugs and Drug Addiction[39]
337 where cocaine seems to be a big city phenomenon with special relevance in Oslo and at the same time within
338 the European context Norway has been shown to have one of the biggest market for
339 amphetamine/methamphetamine[39].

340 Cocaethylene, the main urinary biomarker of cocaine co-consumption with alcohol, and MDMA were
341 identified and quantified only in Oslo. Methylone, ketamine and methoxetamine were found in at least one of
342 the composite samples from Oslo suggesting a sporadic weekend use.

343 This method is also been successfully used for the analysis of other sewage-based samples, such as pooled
344 urine samples and passive sampler extracts. Sample preparation was adapted for each case (SI). It is worth
345 mentioning the importance of the dilution factor for both POCIS and pooled urine samples since the
346 concentration level for the target biomarkers are extraordinary high compared with those typically found in
347 wastewater, being a very suitable samples for suspect screenings.

348 **Table S-5 and S-6** show the results of the target analysis performed on 10 POCIS extracts from the winter of
349 2013 and three pooled urine samples collected from three different music festivals in summer of 2014. The
350 results for the passive sampling devices must be corrected with an exposure factor, however this is outside of
351 the scope of this paper and therefore the results are presented as amount of analyte (ng) per POCIS. The target
352 method was also used for the analysis of the pooled urine samples collected from music festivals.

353 In general the compounds identified in the POCIS do not differ with the compounds obtained and reported in
354 this study for the wastewater samples, however in the pooled urine samples the differences are greater,
355 especially for the amphetamine-like compounds exposed to a lower dilution factor compared with the other
356 sewage-based samples. The low dilution factor makes pooled urine samples a very suitable sample for suspect
357 screening purposes.

358 3.6.2 Suspect Screening

359 The suspect screening database was applied to the pooled urine samples to demonstrate the applicability of
360 these libraries. The aim of this work was to develop a reiterative tool to track the presence of the target
361 compounds in wastewater by complementing the 51 target analytes with new targets identified via suspect
362 screening The 51 compounds were selected as described above, however as shown in **Table 2** not all were
363 present or detected in the sewage-based samples. Suspect screening together with retrospective analysis allows
364 the selection of new compounds, based on detection in the wastewater to be added to the quantitative target

365 method through the use of an authentic reference standard thereby facilitating decision-making and improving
366 cost effectiveness.

367 The continuum data for the three-music festival samples were peak detected using the processing settings
368 described above. Following peak processing UNIFI provided a list of m/z values termed as candidates. The
369 number of candidates for the samples were 11,220, 11,183 and 11,164 for each festival. The acceptance
370 criterion for the tentative candidates is within the tolerance range described in the data processing section.
371 Despite this, in this method the authors defined limit checks broader to perform the final step manually,
372 meaning that after the evaluation of the candidates against the database UNIFI provided a new list with 182,
373 151 and 131 tentative IDs respectively. Table S-7 shows some examples of positive identifications for the ID's
374 fulfilling the criteria consisted of a mass accuracy ± 5 ppm, retention time ± 0.5 min, isotope match ± 5 ppm and
375 a minimum of two detected ions in the HE. Sildenafil, a medicine to treat erectile dysfunction, zolpidem,
376 prescription medication for the treatment of insomnia, and benzocaine and lidocaine, known cocaine cutting
377 agents are interesting for future target analysis. In this case the 24 compounds identified (Table S-7) would
378 move from the level 3 (tentative candidates) to the level 2 (probable structure) in the absence of the reference
379 standards for the final confirmation [33].

380 In order to augment the suspect database used in this study, a non-target approach was used to net purchased
381 NPS; 5/6-APB and N-adamantyl-1-pentylindole-3-carboxamide (commercial names: Benzo Fury and 2NE1-
382 APICA). **Figures S-4 and S-5** illustrate the detection and identification of the 5/6-APB and N-adamantyl-1-
383 pentylindole-3-carboxamide. UNIFI automatically detects the sample components, and subsequently through
384 the accurate mass spectra allows the identification of the unknown candidates. In contrast with the
385 characterization analysis of the classic drugs, the NPS chromatograms are relatively clean since they are
386 purchased in almost pure form without any additive or "cutting agent" as is shown in **Figure S-4 and S-5** where
387 only two peaks are present as the most abundant. As an example, **Figure S-5** shows a chromatogram with the
388 assigned fragments (159.0798 and 131.0488) from the accurate mass ion 176.1065 at 5.2 min.

389 A common fragment approach was used also used as a complementary tool for the identification and
390 subsequently introduction of 14 synthetic cannabinoids into the suspect database. **Figure S-6** shows the total
391 ion chromatogram of the 14 synthetic cannabinoids described in the picture together with the chromatogram of
392 the common fragment m/z 155.0492 belonging to 8 of the synthetic cannabinoids.

393

394

395

396 **4. CONCLUSIONS**

397 A high confidence multi-residue method using auto-SPE-UHPLC-QTOF for the determination of 51 drugs,
398 pharmaceuticals and metabolites was validated and applied to different sewage-base samples. Meanwhile, this
399 analytical procedure is also intended to fulfill the analytical challenge of the detection and reliable
400 identification of NPS appearing in the market through the application of a suspect screening approach
401 augmented by a reiterative process intended to both update the target multi-residue method, and the suspect
402 screening list.

403 The selection of HLB as an extraction sorbent and C18 as the chromatography column provides a broad and
404 generic methodology for the rapid identification of a larger number of compounds dealing with the compromise
405 between elimination of interfering matrix components (using more selective extraction procedures) or the
406 unification of all requested features in just one method. The method has a satisfactory linear dynamic range and
407 the sensitivity. Even though many papers have shown the capability of the HRMS screening techniques just for
408 qualitative purposes, the high selectivity, accurate mass and the information provided by the fragments as mass
409 defect or the isotopic pattern, make this method in a very reliable tool also for the quantification of drugs and
410 emergent substances in wastewater.

411 The described reiterative and dynamic workflow has been designed in order to constantly update the screening
412 levels based on experimental data (**Figure 3** Target analysis can be supplemented by simultaneously screening
413 through the suspect database in order to identify new compound of interest, all in one injection. If a suspect
414 candidate is identified, this candidate can be later confirmed by purchasing the reference standard and this
415 compound can then be incorporated into the target method. Despite not being used in this work, non-target
416 screening is an additional avenue of the described workflow which can enhance the potential of the approach.
417 The application of this method was finally tested with different real samples showing an extensive list of
418 confirmed compounds in different sewage-based samples confirming the broad applicability of the developed
419 method. Two NPS were also introduced into the suspect database together with another 14 synthetic
420 cannabinoids as part of the strategy to expand the library for further identification purposes in the future.

421

422 **ACKNOWLEDGEMENTS**

423 The authors would like to thank Pia Ryrfors, VEAS WWTP, for providing access to the sampling area and the
424 best information regarding the wastewater samples. Jose Antonio Baz Lomba acknowledges the EU
425 International Training Network SEWPROF (Marie Curie-FP7-PEOPLE, grant number 317205) for his Early
426 Stage Researcher grant

427

428 REFERENCES

429

- 430 [1] UNODC, World Drug Report 2014, United Nations Office on Drugs and Crime
431 (UNODC), 2014.
- 432 [2] EMCDDA, European Drug Report 2015: Trends and Developments, European
433 Monitoring Centre for Drugs and Drug Addiction, Lisbon, 2015.
- 434 [3] EMCDDA, New psychoactive substances in Europe. An update from the EU Early
435 Warning System, in: P.O.o.t.E. Union (Ed.), European Monitoring Centre for Drugs and
436 Drug Addiction, Luxembourg, 2015.
- 437 [4] L. Bijlsma, J.V. Sancho, F. Hernandez, W.M. Niessen, Fragmentation pathways of
438 drugs of abuse and their metabolites based on QTOF MS/MS and MS(E) accurate-mass
439 spectra, *Journal of mass spectrometry : JMS*, 46 (2011) 865-875.
- 440 [5] M. Gros, S. Rodriguez-Mozaz, D. Barcelo, Fast and comprehensive multi-residue
441 analysis of a broad range of human and veterinary pharmaceuticals and some of their
442 metabolites in surface and treated waters by ultra-high-performance liquid
443 chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry, *J*
444 *Chromatogr A*, 1248 (2012) 104-121.
- 445 [6] C. Ort, M.G. Lawrence, J. Reungoat, G. Eaglesham, S. Carter, J. Keller,
446 Determining the fraction of pharmaceutical residues in wastewater originating from a
447 hospital, *Water Research*, 44 (2010) 605-615.
- 448 [7] K.V. Thomas, C. Dye, M. Schlabach, K.H. Langford, Source to sink tracking of
449 selected human pharmaceuticals from two Oslo city hospitals and a wastewater
450 treatment works, *J Environ Monit*, 9 (2007) 1410-1418.
- 451 [8] K. Langford, K.V. Thomas, Input of selected human pharmaceutical metabolites
452 into the Norwegian aquatic environment, *Journal of Environmental Monitoring*, 13
453 (2011) 416-421.
- 454 [9] T.L. ter Laak, P.J. Kooij, H. Tolkamp, J. Hofman, Different compositions of
455 pharmaceuticals in Dutch and Belgian rivers explained by consumption patterns and
456 treatment efficiency, *Environ Sci Pollut Res Int*, 21 (2014) 12843-12855.
- 457 [10] D.R. Baker, B. Kasprzyk-Hordern, Spatial and temporal occurrence of
458 pharmaceuticals and illicit drugs in the aqueous environment and during wastewater
459 treatment: New developments, *Science of The Total Environment*, 454-455 (2013) 442-
460 456.
- 461 [11] P. Verlicchi, M. Al Aukidy, A. Jelic, M. Petrović, D. Barceló, Comparison of
462 measured and predicted concentrations of selected pharmaceuticals in wastewater and
463 surface water: A case study of a catchment area in the Po Valley (Italy), *Science of The*
464 *Total Environment*, 470-471 (2014) 844-854.
- 465 [12] A.L. van Nuijs, A. Covaci, H. Beyers, L. Bervoets, R. Blust, G. Verpooten, H.
466 Neels, P.G. Jorens, Do concentrations of pharmaceuticals in sewage reflect prescription
467 figures?, *Environ Sci Pollut Res Int*, 22 (2015) 9110-9118.
- 468 [13] K.V. Thomas, M.J. Reid, What Else Can the Analysis of Sewage for Urinary
469 Biomarkers Reveal About Communities?, *Environmental Science & Technology*, 45
470 (2011) 7611-7612.
- 471 [14] EMCDDA, Wastewater analysis and drugs: a European multi-city study, European
472 Monitoring Centre for Drugs and Drug Addiction, 2015.

473 [15] S. Castiglioni, K.V. Thomas, B. Kasprzyk-Hordern, L. Vandam, P. Griffiths,
474 Testing wastewater to detect illicit drugs: state of the art, potential and research needs,
475 *The Science of the total environment*, 487 (2014) 613-620.

476 [16] A.L.N. van Nuijs, S. Castiglioni, I. Tarcomnicu, C. Postigo, M.L. de Alda, H.
477 Neels, E. Zuccato, D. Barcelo, A. Covaci, Illicit drug consumption estimations derived
478 from wastewater analysis: A critical review, *Science of The Total Environment*, 409
479 (2011) 3564-3577.

480 [17] C. Harman, M. Reid, K.V. Thomas, In situ calibration of a passive sampling device
481 for selected illicit drugs and their metabolites in wastewater, and subsequent year-long
482 assessment of community drug usage, *Environ Sci Technol*, 45 (2011) 5676-5682.

483 [18] I. Gonzalez-Marino, J.B. Quintana, I. Rodriguez, M. Gonzalez-Diez, R. Cela,
484 Screening and selective quantification of illicit drugs in wastewater by mixed-mode
485 solid-phase extraction and quadrupole-time-of-flight liquid chromatography-mass
486 spectrometry, *Analytical chemistry*, 84 (2012) 1708-1717.

487 [19] K.V. Thomas, L. Bijlsma, S. Castiglioni, A. Covaci, E. Emke, R. Grabic, F.
488 Hernandez, S. Karolak, B. Kasprzyk-Hordern, R.H. Lindberg, M. Lopez de Alda, A.
489 Meierjohann, C. Ort, Y. Pico, J.B. Quintana, M. Reid, J. Rieckermann, S. Terzic, A.L.
490 van Nuijs, P. de Voogt, Comparing illicit drug use in 19 European cities through sewage
491 analysis, *The Science of the total environment*, 432 (2012) 432-439.

492 [20] C. Ort, A.L. van Nuijs, J.D. Berset, L. Bijlsma, S. Castiglioni, A. Covaci, P. de
493 Voogt, E. Emke, D. Fatta-Kassinos, P. Griffiths, F. Hernandez, I. Gonzalez-Marino, R.
494 Grabic, B. Kasprzyk-Hordern, N. Mastroianni, A. Meierjohann, T. Nefau, M. Ostman,
495 Y. Pico, I. Racamonde, M. Reid, J. Slobodnik, S. Terzic, N. Thomaidis, K.V. Thomas,
496 Spatial differences and temporal changes in illicit drug use in Europe quantified by
497 wastewater analysis, *Addiction*, 109 (2014) 1338-1352.

498 [21] E. Zuccato, S. Castiglioni, M. Tettamanti, R. Olandese, R. Bagnati, M. Melis, R.
499 Fanelli, Changes in illicit drug consumption patterns in 2009 detected by wastewater
500 analysis, *Drug Alcohol Depend*, 118 (2011) 464-469.

501 [22] F.Y. Lai, R. Bruno, W. Hall, C. Gartner, C. Ort, P. Kirkbride, J. Prichard, P.K.
502 Thai, S. Carter, J.F. Mueller, Profiles of illicit drug use during annual key holiday and
503 control periods in Australia: wastewater analysis in an urban, a semi-rural and a
504 vacation area, *Addiction*, 108 (2013) 556-565.

505 [23] J.R. Archer, P.I. Dargan, H.M. Lee, S. Hudson, D.M. Wood, Trend analysis of
506 anonymised pooled urine from portable street urinals in central London identifies
507 variation in the use of novel psychoactive substances, *Clin Toxicol (Phila)*, 52 (2014)
508 160-165.

509 [24] M.J. Reid, L. Derry, K.V. Thomas, Analysis of new classes of recreational drugs in
510 sewage: synthetic cannabinoids and amphetamine-like substances, *Drug Test Anal*, 6
511 (2014) 72-79.

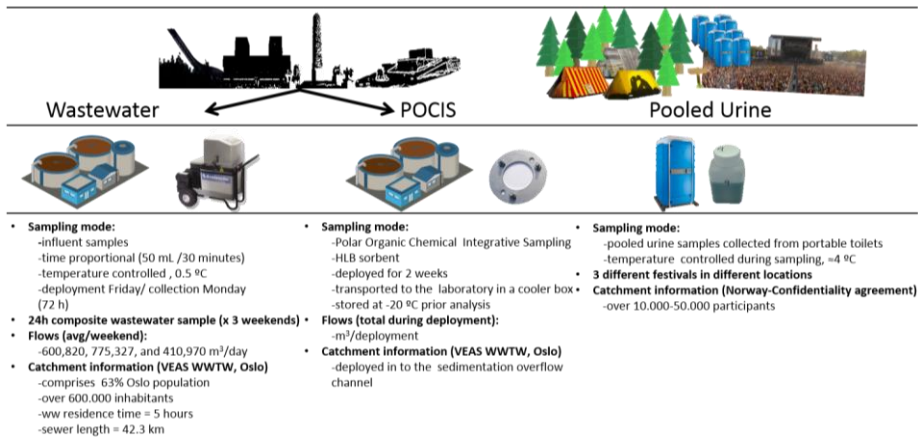
512 [25] J. Kinyua, A. Covaci, W. Maho, A.K. McCall, H. Neels, A.L. van Nuijs, Sewage-
513 based epidemiology in monitoring the use of new psychoactive substances: Validation
514 and application of an analytical method using LC-MS/MS, *Drug Test Anal*, (2015) n/a-
515 n/a.

516 [26] M. Ibáñez, J.V. Sancho, L. Bijlsma, A.L.N. van Nuijs, A. Covaci, F. Hernández,
517 Comprehensive analytical strategies based on high-resolution time-of-flight mass
518 spectrometry to identify new psychoactive substances, *TrAC Trends in Analytical
519 Chemistry*, 57 (2014) 107-117.

520 [27] F. Hernández, M. Ibáñez, R. Bade, L. Bijlsma, J.V. Sancho, Investigation of
521 pharmaceuticals and illicit drugs in waters by liquid chromatography-high-resolution
522 mass spectrometry, *TrAC Trends in Analytical Chemistry*, 63 (2014) 140-157.
523 [28] M. Ibanez, L. Bijlsma, A.L. van Nuijs, J.V. Sancho, G. Haro, A. Covaci, F.
524 Hernandez, Quadrupole-time-of-flight mass spectrometry screening for synthetic
525 cannabinoids in herbal blends, *Journal of mass spectrometry : JMS*, 48 (2013) 685-694.
526 [29] U.Ö. B. Magnusson, *Eurachem Guide: The Fitness for Purpose of Analytical*
527 *Methods – A Laboratory Guide to Method Validation and Related Topics*, ISBN 978-
528 91-87461-59-0 (2nd ed. 2014).
529 [30] Commission Decision 2002/657/EC of 12 August 2002 implementing Council
530 Directive 96/23/EC concerning to the performance of analytical methods and the
531 implementation of the results, *Official Journal of the European Communities*, (2002) 8–
532 62.
533 [31] R. Lee, M. Roberts, A. Paccou, M. Wood, Development of a new UPLC/MS
534 method for systematic toxicological analysis, *Waters Corp. Application Note*
535 72002905EN, (2009).
536 [32] M. Krauss, H. Singer, J. Hollender, LC–high resolution MS in environmental
537 analysis: from target screening to the identification of unknowns, *Analytical and*
538 *bioanalytical chemistry*, 397 (2010) 943-951.
539 [33] E. Schymanski, H. Singer, J. Slobodnik, I. Ipolyi, P. Oswald, M. Krauss, T.
540 Schulze, P. Haglund, T. Letzel, S. Grosse, N. Thomaidis, A. Bletsou, C. Zwiener, M.
541 Ibáñez, T. Portolés, R. de Boer, M. Reid, M. Onghena, U. Kunkel, W. Schulz, A.
542 Guillon, N. Noyon, G. Leroy, P. Bados, S. Bogialli, D. Stipaničev, P. Rostkowski, J.
543 Hollender, Non-target screening with high-resolution mass spectrometry: critical review
544 using a collaborative trial on water analysis, *Analytical and bioanalytical chemistry*,
545 (2015) 1-19.
546 [34] M.J. Reid, J.A. Baz-Lomba, Y. Ryu, K.V. Thomas, Using biomarkers in
547 wastewater to monitor community drug use: A conceptual approach for dealing with
548 new psychoactive substances, *Science of the Total Environment*, 487 (2014) 651-658.
549 [35] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the
550 assessment of matrix effect in quantitative bioanalytical methods based on HPLC-
551 MS/MS, *Analytical chemistry*, 75 (2003) 3019-3030.
552 [36] L. Bijlsma, E. Beltrán, C. Boix, J.V. Sancho, F. Hernández, Improvements in
553 analytical methodology for the determination of frequently consumed illicit drugs in
554 urban wastewater, *Analytical and bioanalytical chemistry*, (2014) 1-12.
555 [37] A.L. van Nuijs, A. Gheorghe, P.G. Jorens, K. Maudens, H. Neels, A. Covaci,
556 Optimization, validation, and the application of liquid chromatography-tandem mass
557 spectrometry for the analysis of new drugs of abuse in wastewater, *Drug Test Anal*, 6
558 (2014) 861-867.
559 [38] S. Castiglioni, L. Bijlsma, A. Covaci, E. Emke, F. Hernandez, M. Reid, C. Ort,
560 K.V. Thomas, A.L. van Nuijs, P. de Voogt, E. Zuccato, Evaluation of uncertainties
561 associated with the determination of community drug use through the measurement of
562 sewage drug biomarkers, *Environ Sci Technol*, 47 (2013) 1452-1460.
563 [39] The Drug Situation in Norway. Annual report to the European Monitoring Centre
564 for Drugs and Drug Addiction – EMCDDA Norwegian Institute for Alcohol and Drug
565 Research (SIRUS), Oslo, 2015.

568 **Figures and Tables**

569



570

571 **Figure 1**

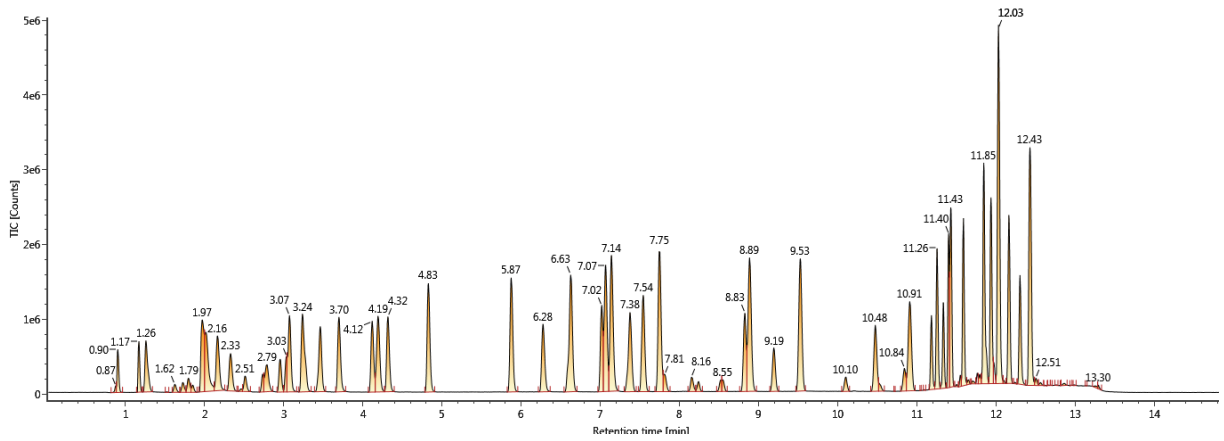
572 **Different sewage-based samples used in this study**

573

574

575

576



577

578

Figure 2

579

2D mass chromatogram of the low energy channel (6eV ESI+) of a 100 ng/mL standard mix solution with all

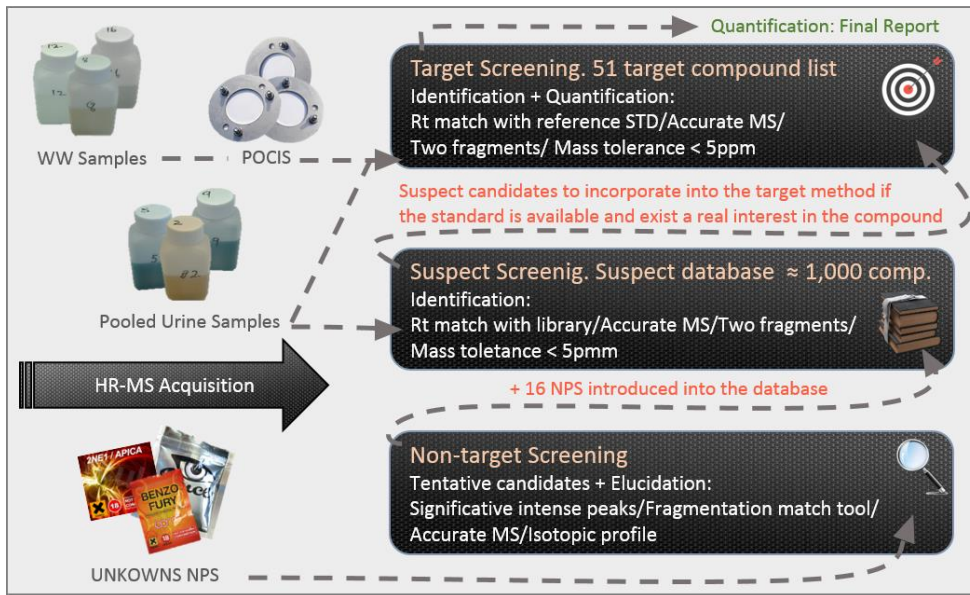
580

the target compounds, UNIFI (Waters Corporation, Milford MA, USA)

581

582

583



584

585 **Figure 3. Illustrative workflow diagram for the three different approaches developed in this study.**

586

587

Compound	Internal Standard	t_R	Precursor ion	Product ion	Linear range
			[M + H] ⁺	(m/z)	(ng mL ⁻¹)
Morphine	Atenolol-d ₇	1.17	286.14376	201.0910	0,5-100
Hydroxycotinine	Atenolol-d ₇	1.17	193.09714	134.0595	0,5-400
Atenolol	Atenolol-d ₇	1.26	267.1703	190.0863	0,5-200
Salbutamol	Atenolol-d ₇	1.28	240.1594	145.0648	1-100
p-Hydroxymethamphetamine	Atenolol-d ₇	1.31	166.12263	135.0798	2-100
Paracetamol	Atenolol-d ₇	1.62	152.07059	110.0601	2-400
Cathinone	Methcathinone-d ₃	1.75	150.09133	132.0808	5-400
Gabapentin	Gabapentin-d ₁₀	1.8	172.13319	154.1227	5-200
Pseudoephedrine	Methcathinone-d ₃	1.96	166.12263	133.0886	5-400
Methcathinone (Ephedrone)	Methcathinone-d ₃	1.98	164.10698	146.0971	1-400
Methylone	Methylone-d ₃	2.16	208.09681	160.0757	0,25-400
6-MAM	Methylone-d ₃	2.29	328.15432	165.0699	0,5-400
Amphetamine	Amphetamine-d ₅	2.41	136.11207	91.0543	5-400
MDA	MDA-d ₃	2.47	180.10189	163.0754	10-200
PMA	MDMA-d ₃	2.53	166.12263	91.0543	2-400
Methamphetamine	Methamphetamine-d ₅	2.72	150.12771	119.0856	2-200
MDMA	MDMA-d ₃	2.75	194.11754	163.0754	2-400
Dehydronorketamine	Norketamine-d ₄	2.89	222.06801	205.0415	1-400
PMMA	MDMA-d ₃	2.98	180.13828	121.0648	2-400
Benzoylcegonine	Benzoylcegonine-d ₃	3.07	290.13867	168.1020	0,25-400
Mephedrone (4-MMC)	Mephedrone-d ₃	3.19	178.12263	160.1127	2-400
Norketamine	Norketamine-d ₄	3.21	224.08366	125.0153	5-200
Ketamine	Ketamine-d ₄	3.45	238.09931	125.0153	0,5-400
4-MEC	Ketamine-d ₄	3.7	192.13828	174.1278	0,5-400
Methoxetamine	Metoprolol-d ₇	4.12	248.16449	121.0648	0,25-400
Metoprolol	Metoprolol-d ₇	4.19	268.1907	116.1070	0,25-400
Methylphenidate	Metoprolol-d ₇	4.32	234.14884	84.0808	0,25-400
Cocaine	Cocaine-d ₃	4.83	304.15432	182.1176	0,25-200
Cocacethylene	Cocaine-d ₃	5.87	318.16997	196.1333	0,25-400
Propranolol	Fentanyl-d ₅	6.28	260.16449	116.1070	0,25-200
Fentanyl	Fentanyl-d ₅	6.63	337.22742	188.1434	0,25-400
AH-7921	Fentanyl-d ₅	7.02	329.11818	284.0611	0,25-400
Citalopram	Buprenorphine-d ₄	7.07	325.17105	109.0449	0,25-200
Midazolam	Buprenorphine-d ₄	7.14	326.08547	291.1167	0,25-400
Buprenorphine	Buprenorphine-d ₄	7.38	468.31081	414.2633	0,25-400
Carbamazepine	Buprenorphine-d ₄	7.54	237.10223	194.0965	0,25-200
EDDP	Atomoxetine-d ₇	7.75	278.19031	234.1277	0,25-400
Atomoxetine	Atomoxetine-d ₇	7.81	256.16958	148.1103	1-200
Nitrazepam	Oxazepam-d ₅	8.16	282.08731	236.0945	1-400
Oxazepam	Oxazepam-d ₅	8.22	287.05817	241.0528	2-400
Clonazepam	Oxazepam-d ₅	8.5	316.04833	270.0555	1-400
Lorazepam	Oxazepam-d ₅	8.6	321.0192	275.0138	5-400
Alprazolam	Oxazepam-d ₅	8.83	309.09014	281.0715	0,25-200
Methadone	Methadone-d ₃	8.89	310.21652	265.1587	0,25-400
Flunitrazepam	Diazepam-d ₅	9.19	314.09353	268.1007	1-400
Etizolam	Diazepam-d ₅	9.53	343.07786	314.0391	0,25-200
Phenazepam	Diazepam-d ₅	10.1	348.97382	196.1706	2-400
Diazepam	Diazepam-d ₅	10.91	285.07891	193.0886	0,25-200
Diclofenac	Diazepam-d ₅	11.4	296.02395	215.0496	5-400
THC-OH	THC-OH-d ₃	11.83	331.22675	313.2162	5-400
THC-COOH	THC-COOH-d ₃	11.85	345.20602	299.2009	5-400

593

Table 2

594

Loads (mg day⁻¹ 1000 inhabitants⁻¹) of detected analytes in influent wastewater samples from the Oslo (mean

595

± SD) and Trondheim (mean, n=1) WWTPs

596

a founded it at least one of the daily samples (not presented in all)

Analyte	Oslo, February 2014						Trondheim, Spring 2014					
	1st weekend		2nd weekend		3rd weekend		Friday	Saturday	Friday	Saturday	Friday	Saturday
							28.02.14	08.03.14	21.03.14	29.03.14	04.04.14	12.04.14
Morphine	48.2	±12.3	41.8	±13.4	47.5	±9.2	6.1	7.2	8.7	5.5	13.3	12.3
Hydroxycotinine	3873.8	±616.9	1919.1	±204.9	2155.9	±243.6	239.5	331.7	500.1	577.7	575.3	636.3
Atenolol	165.7	±22.0	134.3	±21.1	136.2	±6.5	45.0	75.3	53.3	53.2	95.8	77.1
Paracetamol	6323.7	±1026.7	6335.2	±153.2	4210.4	±468.2	485.6	1129.3	1703.1	1481.7	2355.3	2209.2
Methylone ^a	11.8	-	nd	-	nd	-	nd	nd	nd	nd	nd	nd
6-MAM	25.2	±10.5	6.0	±10.4	2.8	±2.4	8.0	16.1	25.3	23.7	32.6	39.2
Amphetamine	51.5	±10.8	38.1	±7.6	51.1	±4.1	76.5	124.1	91.2	53.2	91.8	56.9
MDMA	75.5	±65.5	25.7	±22.3	26.8	±23.8	nd	nd	nd	nd	nd	nd
Methamphetamine	<LOQ	-	26.3	±9.7	25.8	±2.4	14.2	26.3	11.6	35.2	50.1	49.6
Benzoylcegonine	214.3	±86.8	216.3	±24.1	240.9	±59.1	3.5	14.5	5.8	9.0	9.0	12.2
Ketamine ^a	nd	-	5.3	±9.1	nd	-	nd	nd	nd	nd	nd	nd
Methoxetamine ^a	0.8	±1.3	nd	-	nd	-	nd	nd	nd	nd	nd	nd
Metoprolol	836.2	±87.5	859.1	±183.9	857.3	±64.0	123.0	125.6	141.8	146.3	280.1	250.2
Methylphenidate	nd	-	nd	-	nd	-	0.6	1.2	2.7	1.3	2.7	3.0
Cocaine	92.5	±35.5	77.1	±18.5	88.7	±23.6	0.8	2.1	3.1	2.0	3.4	4.0
Cocaethylene	7.3	±6.7	9.8	±1.3	6.1	±1.7	nd	nd	nd	nd	nd	nd
Propranolol	3.5	±0.7	14.8	±1.5	0.8	±0.1	4.3	13.9	20.8	13.0	22.7	26.7
Galopram	44.0	±9.1	49.3	±7.7	44.8	±0.9	32.2	43.9	14.5	12.3	27.5	17.4
Buprenorphine	nd	-	nd	-	nd	-	214.5	292.5	96.7	81.8	183.5	115.7
Carbamazepine	258.3	±29.2	294.6	±16.6	248.0	±27.1	52.1	52.2	35.0	69.2	116.7	112.7
EDDP	48.5	±9.2	36.3	±3.5	18.6	±1.9	10.3	11.6	7.2	7.1	13.9	8.6
Oxazepam	153.2	±32.3	125.0	±25.9	144.0	±6.5	72.6	119.8	69.3	94.7	141.4	140.9
Alprazolam	nd	-	nd	-	nd	-	7.5	798.8	462.0	631.0	942.5	939.1
Methadone	13.4	±2.3	16.8	±4.1	14.5	±2.3	1.1	2.1	3.7	1.8	3.2	3.7

597

598

599

Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF

J.A. Baz-Lomba^{ab*}, Malcolm J. Reid^a, Kevin V. Thomas^a

^aNorwegian Institute for Water Research, Gaustadalléen 21, NO-0349 Oslo, Norway

^bFaculty of Medicine, University of Oslo, PO box 1078 Blindern, 0316 Oslo, Norway

* Corresponding author email: jba@niva.no; Tel: +47 98215460

Experimental Section

Reagents

Sewage-based samples

Extraction procedure

Figure S-1: Influence of silanisation on the recovery

Figure S-2: Recovery of the studied compounds after the evaporation to dryness

Figure S-3: Effect of the loading volume on the matrix effect

Figure S-4: Detection and identification of N-adamantyl-1-pentylindole-3- carboxamide

Figure S-5: Detection and identification 6-APB/ 5-APB

Figure S-6: Total ion chromatogram for the synthetic cannabinoids

Table S-1: SPE-DEX procedure

Table S- 2: Experimental parameters used for the validation and quantification

Table S-3: Method validation in influent wastewater (n=6)

Table S-4: Stability of the spiked analytes in WW

Table S-5: Results obtained for POCIS

Table S-6: Results for target compounds obtained from the pooled urine samples

Table S-7: Occurrence of the suspect candidates in pooled urine samples

Experimental Section

Reagents

The illicit drugs, pharmaceuticals and their metabolites reference substances selected for target analysis were cocaine, cocaethylene, benzoylecgonine, amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDA), 3,4-methylenedioxyethylamphetamine (MDMA or ecstasy), para-hydroxymethamphetamine, para-methoxyamphetamine (PMA), para-methoxy-N-methylamphetamine (PMMA), ketamine, dehydronorketamine, norketamine, methoxetamine, pseudoephedrine, cathinone, methcathinone (ephedrone), methylone, 4'-methyl-N-ethylcathinone (4-MEC), mephedrone (4-MMC), methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), morphine, 6-monoacetylmorphine (6-MAM), fentanyl, buprenorphine, AH-7921, hydroxycotinine, atenolol, salbutamol, gabapentin, paracetamol, metoprolol, methylphenidate, propranolol, citalopram, atomoxetine, carbamazepine, midazolam, nitrazepam, clonazepam, lorazepam, oxazepam, alprazolam, flunitrazepam, etizolam, phenazepam, diazepam, diclofenac, etizolam, carbamazepine, Δ -9-tetrahydrocannabinol (THC), 11-hydroxy- Δ -9-tetrahydrocannabinol (OH-THC), 11-nor-9-carboxy- Δ -9-tetrahydrocannabinol (THC-COOH), which were obtained from Nerliens Meszansky (Oslo, Norway) as solutions in methanol (MeOH) or acetonitrile (ACN) at concentrations of 1 mg mL⁻¹. Standard solutions of each compound were made at 100 ng mL⁻¹ in methanol and then diluted into a final mix solution to a concentration of 1 ng mL⁻¹.

Synthetic cannabinoids and their selected biomarkers, JWH-073, XLR-11, UR-144, AM-2201, JWH-018, MAM-2201, JWH-122, 5-3-1-Naphthoyl-1H-indol-1-yl-pentanoic acid (JWH-018 N-pentanoic acid), 1-5-hydroxypentyl-1H-indol-3-yl-naphthalen-1-yl-methanone (JWH 018 N-5-hydroxypentyl), 4-3-1-naphthoyl-1H-indol-1-yl-butanoic acid (JWH-073 N-butanoic acid), 1-4-hydroxybutyl-1H-indol-3-yl-naphthalen-1-yl-methanone (JWH-073 N-4-hydroxybutyl), 1-5-hydroxypentyl-1H-indol-3-yl-4-methylnaphthalen-1-yl-methanone (JWH-122 N-5-hydroxypentyl), 1-5-fluoro-4-hydroxypentyl-1H-indol-3-yl-naphthalen-1-yl-methanone (AM-2201 N-4-hydroxypentyl), 1-5-hydroxypentyl-1H-indol-3-yl-4-methoxyphenyl-methanone (RCS-4 N-5-hydroxypentyl) were obtained from Chiron (Trondheim, Norway) in solutions of 1 mL at 50 μ g mL⁻¹ in MeOH or ACN.

Benzo Fury, 1-benzofuran-6-ylpropan-2-amine (6-APB) or 1-benzofuran-5-ylpropane-2-amine (5-APB), and 2NE1/APICA, N-adamantyl-1-pentylindole-3-carboxamide were purchased through online website.

Deuterated standards were purchased from Nerliens Meszansky as solutions of 100 ng mL⁻¹ in MeOH or ACN and were used as surrogate isotope labelled internal standards (ILIS) for quantification: atenolol-d7, atomoxetine-d7, diclofenac-d4, gabapentin-d10, metoprolol-d7, pregabalin-d6, benzoylecgonine-d3, cocaine-d3, ketamine-d4, norketamine-d4, mephedrone-d3, methcathinone-d3 (ephedrone-d3), methylone-d3, pseudoephedrine-d3, buprenorphine-d4, diazepam-d5, oxazepam-d5, fentanyl-d5, methadone-d3, amphetamine-d5, MDA-d5, MDMA-d5, methamphetamine-d5, THC-OH-d3, THC-COOH-d3. Final standard mix solution was made in methanol at 1 ng mL⁻¹. Finally all the standard solutions were stored in amber glass bottles at -20 °C.

The ultrapure water was obtained by purifying demineralized water in an Elga Maxima Ultrapure Water purification system (Elga, Lane End, UK). Ammonium formate (for mass spectroscopy, \geq 99.0%), HPLC-grade formic acid (eluent additive for LC-MS) and UHPLC-grade water, MeOH and ACN were acquired from Sigma-Aldrich, Fluka for HPLC (Oslo, Norway)

Sewage-based samples

Influent samples were collected from two wastewater treatment plants (WWTWs); VEAS WWTW in Oslo and Ladehammeren and Høvringen WWTWs in Trondheim Norway.

Nine influent wastewater composite samples were collected during three consecutive weekends from VEAS sewage treatment plant, Oslo Norway, in February 2014. An ISCO Avalanche Portable Refrigerated Sampler (Lincoln, NE, USA) was used to collect the composite samples (50 mL, time proportional) every 30 minutes for the duration of the sampling period. Maximum storage time for the daily composite samples was 3 days at 0.5°C in polypropylene bottles. The composite samples were transported every Monday in cooler bags and stored at -20 °C until analysis to minimize the degradation of the analytes. The catchment area of VEAS is comprised of 63% of the Oslo population, around 600,000 inhabitants. The total length of the tunnel is 42.3 km and the mean residence time in the sewer system is 5 hours (VEAS annual report 13'). The mean influent flow rate for each weekend was 600,820, 775,327, and 410,970 m³/day, which is higher than the annual mean (273,196 m³ day⁻¹ in 2013) and was due to increased precipitation during the sampling campaign.

Six influent 24-hour composite wastewater samples were collected during February, March and April of 2014 in two different sewage treatment plants located in Trondheim (Norway). Ladehammeren and Høvringen WWTPs treat the sewage from the city with an estimated population of 180,000 inhabitants. Samples were received frozen and analysed upon reception in the laboratory. Mean influent flow rates are described in the results section.

The robustness of this work was also examined with the analysis of different sewage-based samples. The "Pharmaceuticals" version of the POCIS was made up of Oasis HLB sandwiched between polyethersulphone membranes as described in literature¹. POCIS were deployed in the sedimentation overflow channel at VEAS, the Oslo WWTP, for two weeks. The study and collection of the pooled urine samples was conducted in Norway during the course of three different music festivals. Portable toilets were available to be used by the festival participants anonymously and no data on the number of users was collected.

Extraction procedure

The HLB extraction disks were conditioned by washing and rinsing with methanol (10 mL) and water (10 mL). Samples were automatically loaded and filtered (Fast Flow Pre-Filters, Horizon Technology) onto the disks at a flow rate of 100 mL min⁻¹, and then the disks were washed 4 times with 5% methanol in water and dried under vacuum for 10 minutes.

Then the samples were loaded directly onto the disk from the sample bottle and prior elution, the disk is air-dried under vacuum providing a shorter operational time. The analytes were finally eluted into a silanised glass vial with 2 cycles of 5% ammonium hydroxide in methanol and 2 cycles of 5% acetic acid in methanol. The total program time was approximately 25 minutes. In general this system is more simple, reproducible and cost-effective than the SPE cartridges.

The final extracts were evaporated to around 100 µL under a gentle nitrogen stream at 35 °C and reconstructed in 400 µL of 25% methanol aqueous solution. An aliquot was centrifuged and then 5 µL were injected into the UHPLC-QTOF. The general procedure is also described in the Table S-1.

The “Pharmaceuticals” version of the POCIS were deployed in the wastewater treatment plant and subsequently the sorbent was removed and introduced in an empty SPE cartridge with Mili-Q water, washed twice with 6 mL of 5 % methanol in water and finally eluted with 6 mL of 0.5% ammonium hydroxide in methanol and 6 mL of 0.5% acetic in methanol. The eluent was evaporated under a stream of nitrogen and diluted to 1.5 mL with 13% methanol in water. 100 ng of the ILIS mix was added during the washing step.

The pooled urine samples were firstly homogenized and then centrifuged during 20 minutes at 2500 rpm. 5 mL of the supernatant was collected, spiked with 100 ng of the ILIS mix and introduced into the HLB cartridge. Washed with 2x6 mL 5 % methanol in water, the elution was finally done with 6 mL of 0.5% ammonium hydroxide in methanol and 6 mL of 0.5% acetic in methanol. The eluent was evaporated under a stream of nitrogen and diluted to 1 mL with 13% methanol in water.

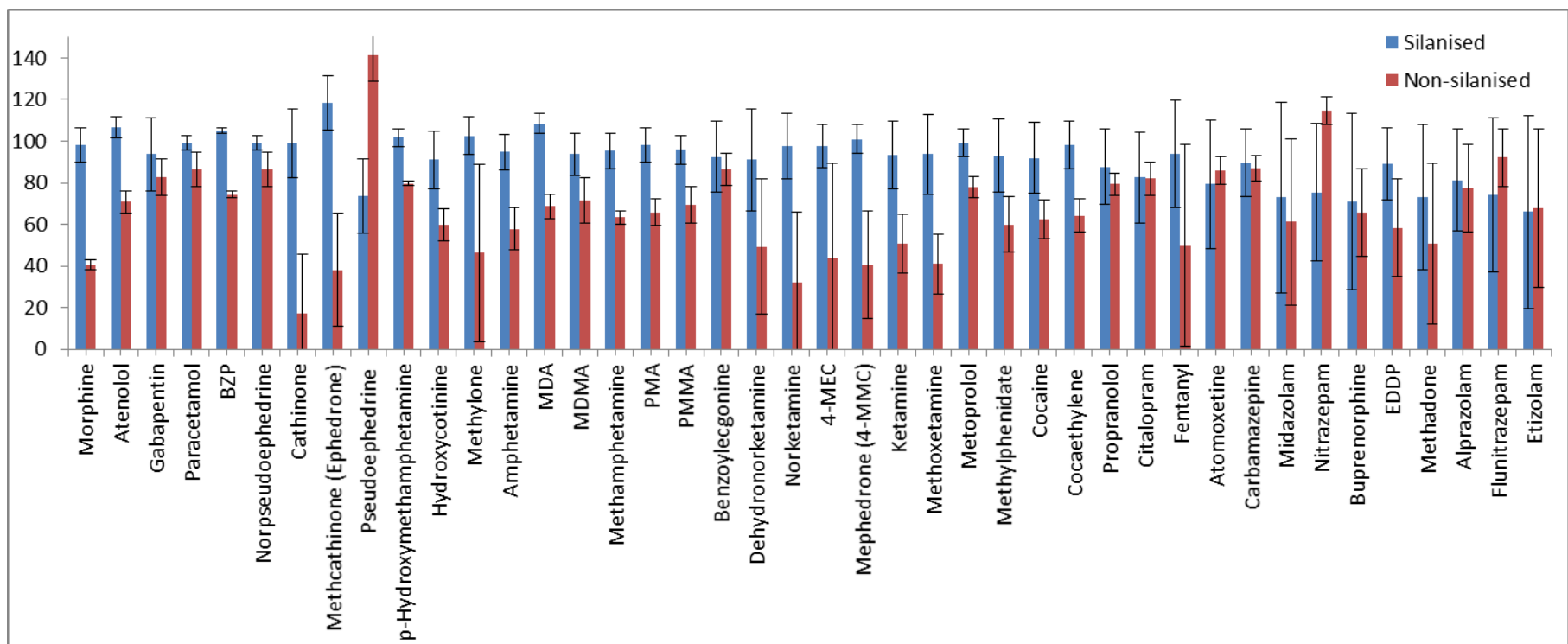


Figure S-1
 Influence of silanisation on the recovery (%) of the studied compounds during the evaporation in the SPE extract vials. Recovery of 100 mL of MiliQ water spiked with 100 ng of the standard mix solution using HLB cartridges (n=3)

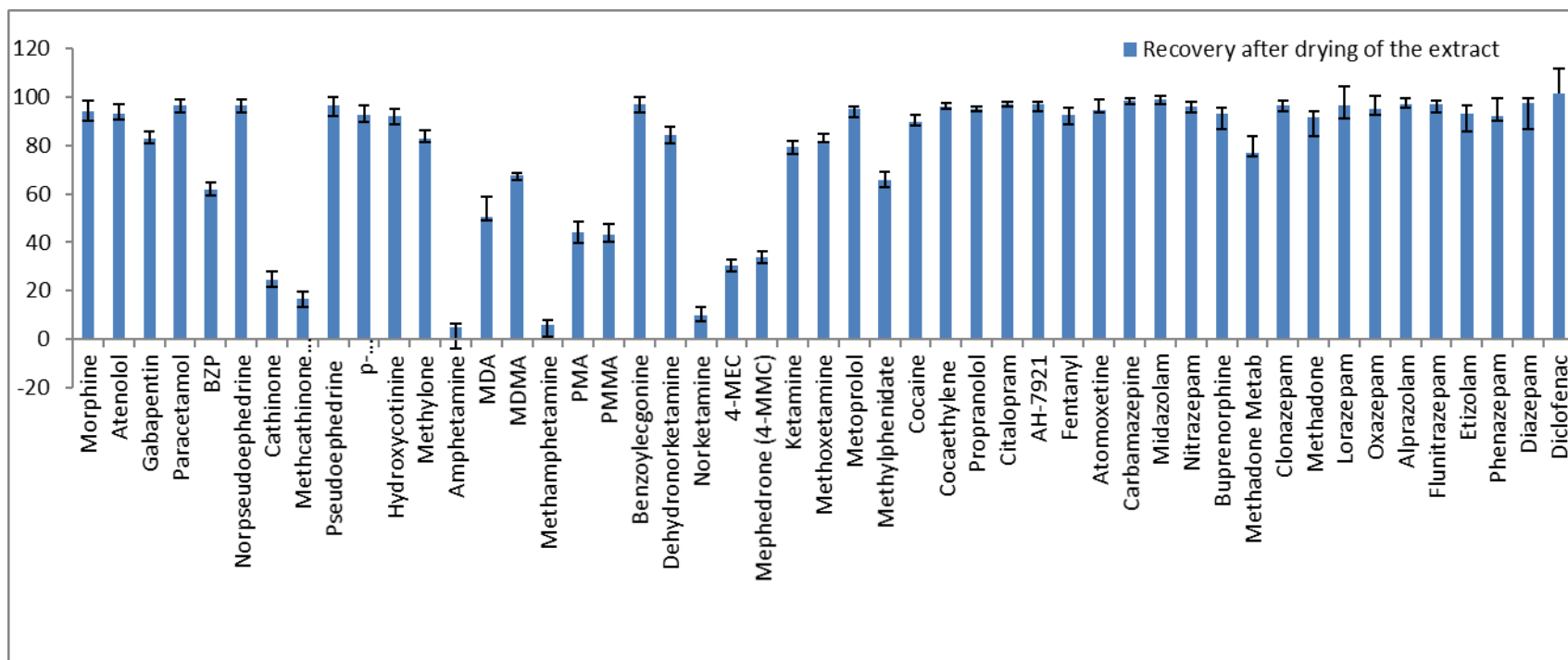


Figure S-2
 Recovery of the studied compounds after the evaporation to dryness at 50 °C of a 5 mL methanol solution with 100 ng of the mix standard solution (n=3)

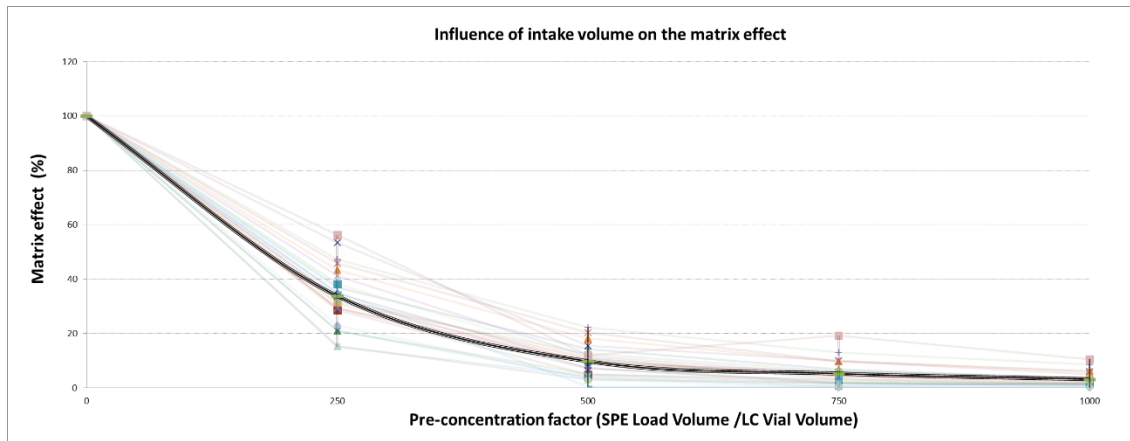


Figure S-3
 Effect of the loading volume on the matrix effect. Matrix suppression average for 25 ILIS spiked into the extracts of 3 different wastewater samples. The loading volumes were 100, 200, 300, and 400 mL and the final extract was reconstituted volume of 400 μ L

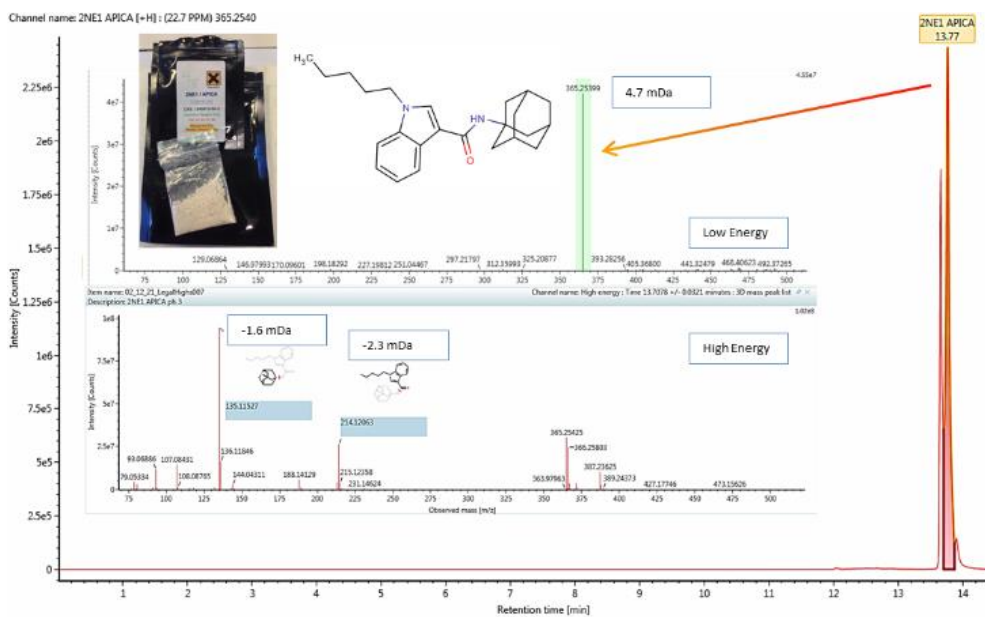


Figure S-1
 Detection and identification of N-adamantyl-1-pentylindole-3-carboxamide in a sample purchased on internet under the name of "2NE1/APICA". Extracted ion chromatogram of the 2NE1/APICA powder sample and the spectra in low and high energy time-of-flight. Possible fragment structures assigned manually by UNIFI

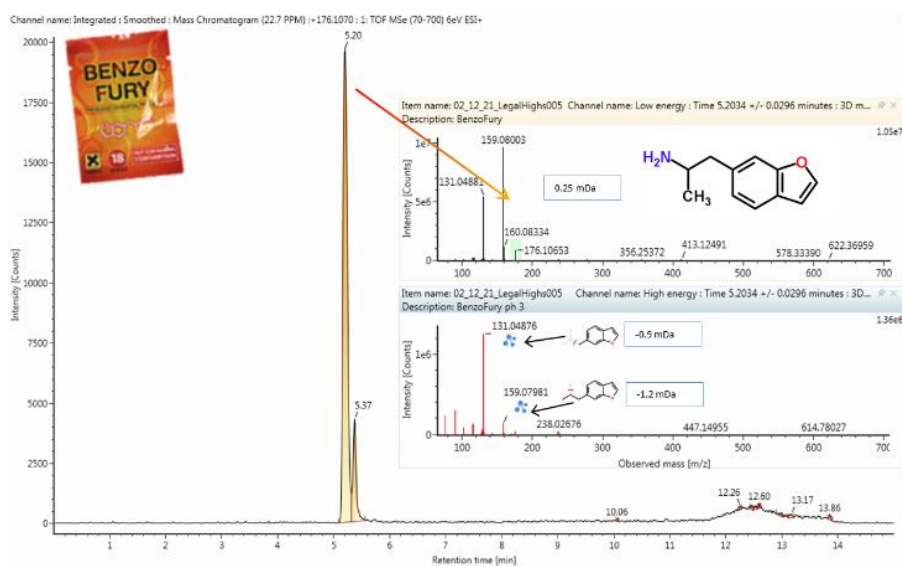
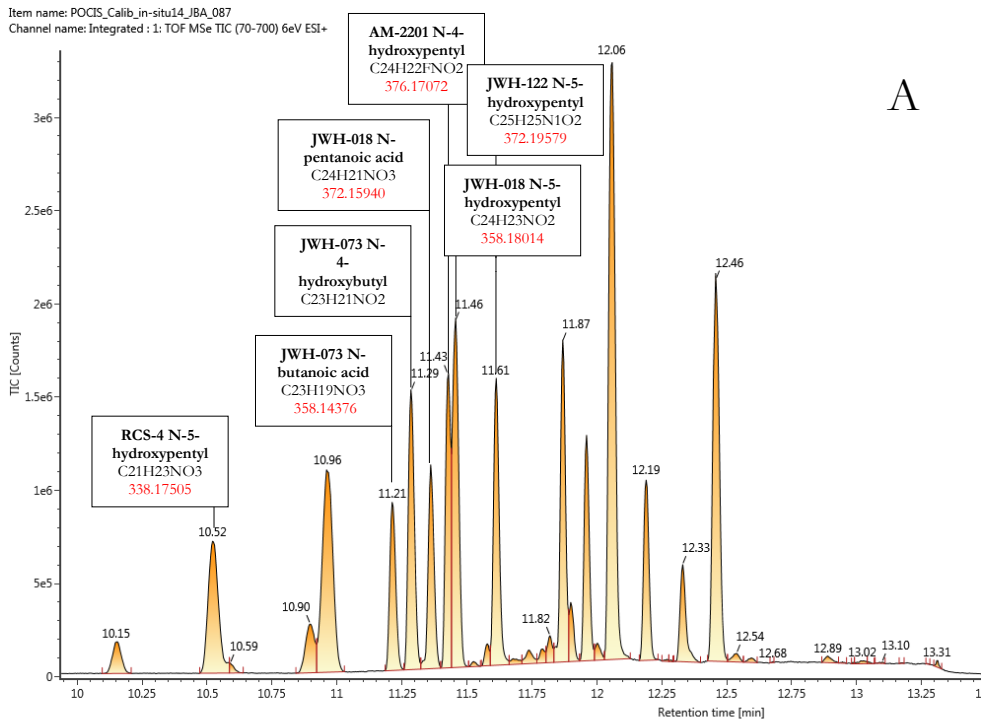
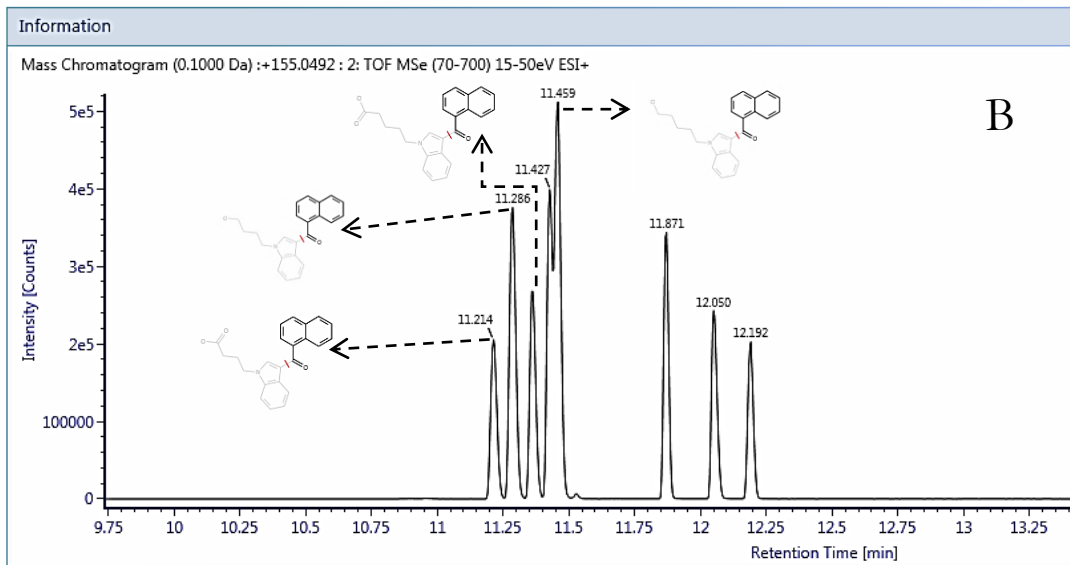


Figure S-2
 Detection and identification of 6-(2-aminopropyl)benzofuran (6-APB)/ 5-APB (5-(2-aminopropyl)benzofuran (5-APB) in a sample purchased on internet under the name of “Benzo Fury”. Extracted ion chromatogram of the Benzo Fury tablet sample and the spectra in low and high energy time-of-flight. Possible fragment structures assigned manually by UNIFI



A



B

Figure S-6
Total ion chromatogram for the 14 synthetic cannabinoids of a standard mix at 250 ng/mL with the description of the main metabolites A). Common fragment (155.0492) chromatogram from four synthetic cannabinoids and their four metabolites B)

Table S-1
SPE-DEX procedure

Step	Solvent	Soak time	Dry time
Preconditioning	Methanol (2 times)	20 s	10 s
	Water	20 s	10 s
	Water	20 s	0 s
Sample loading	-	-	-
Washing	5% methanol/water(4 times)	10 s	30 s
Disk drying	-	-	10 min
Eluting	5% ammonium hydroxide in methanol	30 s	30 s
	5% ammonium hydroxide in methanol	30 s	2 min
	5% acetic acid in methanol	30 s	30 s
	5% acetic acid in methanol	30 s	2 min

Table S- 2

Experimental parameters used for the validation and quantification of the target compounds: chromatographic retention times, exact mass of the protonated target compounds, validated linear range, correlation coefficient, mass error and the results of the instrumental performance

Compound	t_R	Precursor ion $[M + H]^+$	Linear range (ng mL ⁻¹)	Linearity R ²	Mass Error ^a (mDa/ppm)	Accuracy (%)				Precision (RSD%)			
						Intra-day		Inter-day		Intra-day		Inter-day	
						Low	High	Low	High	Low	High	Low	High
Morphine	1.17	286.14376	0,5-100	0.995	-0.08/-0.29	94	101	94	101	1	1	3	2
Hydroxycotinine	1.17	193.09714	0,5-400	0.999	0.09/046	92	97	88	98	3	2	5	5
Atenolol	1.26	267.1703	0,5-200	1.000	-0.05/-0.2	108	99	108	99	3	0	3	0
Salbutamol	1.28	240.1594	1-100	0.995	-0.17/-0.67	108	100	107	99	2	1	5	2
p-Hydroxymethamphetamine	1.31	166.12263	2-100	0.998	-0.17/-0.99	96	102	99	101	7	2	7	2
Paracetamol	1.62	152.07059	2-400	0.998	0/-0.02	98	101	104	101	3	2	9	5
Cathinone	1.75	150.09133	5-400	0.991	-0.08/-0.52	93	104	92	104	3	2	3	1
Gabapentin	1.8	172.13319	5-200	0.995	-0.04/-0.26	110	102	108	99	3	1	2	6
Pseudoephedrine	1.96	166.12263	5-400	0.998	-0.31/-2.07	96	99	94	99	9	1	8	4
Methcathinone (Ephedrone)	1.98	164.10698	1-400	0.998	-0.35/-2.49	93	100	96	100	1	0	3	0
Methylone	2.16	208.09681	0,25-400	0.999	0.03/0.12	110	100	109	100	4	0	3	0
6-MAM	2.29	328.15432	0,5-400	0.998	-0.01/-0.05	95	102	98	101	3	1	4	0
Amphetamine	2.41	136.11207	5-400	0.993	-0.15/-1.31	88	104	98	104	5	1	12	1
MDA	2.47	180.10189	10-200	0.994	0.04/0.29	109	104	108	103	6	4	6	4
PMA	2.53	166.12263	2-400	0.999	-0.42/-2.59	110	100	107	100	2	1	5	1
Methamphetamine	2.72	150.12771	2-200	0.999	-0.16/-1.10	105	101	104	100	4	1	5	1

MDMA	2.75	194.11754	2-400	0.999	-0.03/-0.16	100	100	100	100	4	1	3	1
Dehydronorketamine	2.89	222.06801	1-400	0.998	0.22/0.98	106	99	108	97	7	0	7	2
PMMA	2.98	180.13828	2-400	1.000	-0.42/-2.59	101	100	100	100	3	0	3	1
Benzoylcgonine	3.07	290.13867	0,25-400	0.999	0.01/0.07	94	101	95	101	2	0	1	0
Mephedrone (4-MMC)	3.19	178.12263	2-400	0.999	-0.14/-0.86	107	99	105	99	8	0	8	0
Norketamine	3.21	224.08366	5-200	0.999	0.12/0.58	109	102	104	101	4	1	7	6
Ketamine	3.45	238.09931	0,5-400	1.000	-0.35/-2.49	98	99	97	99	3	1	4	1
4-MEC	3.7	192.13828	0,5-400	1.000	-0.09/-0.48	97	99	98	99	2	1	2	1
Methoxetamine	4.12	248.16449	0,25-400	1.000	-0.06/-0.21	105	99	105	99	7	0	5	0
Metoprolol	4.19	268.1907	0,25-400	0.999	-0.16/-0.61	102	100	101	100	1	1	2	1
Methylphenidate	4.32	234.14884	0,25-400	1.000	0/-0.05	98	100	94	100	3	1	5	1
Cocaine	4.83	304.15432	0,25-200	1.000	0/0.03	97	101	97	101	2	0	1	0
Cocaethylene	5.87	318.16997	0,25-400	1.000	0.04/-0.13	96	102	96	101	1	1	1	1
Propranolol	6.28	260.16449	0,25-200	0.998	-0.04/-0.13	105	99	105	94	2	1	2	6
Fentanyl	6.63	337.22742	0,25-400	1.000	-0.24/-0.69	107	99	105	99	4	0	5	1
AH-7921	7.02	329.11818	0,25-400	1.000	-0.1/-0.33	110	99	113	100	4	0	5	2
Citalopram	7.07	325.17105	0,25-200	0.997	-0.14/-0.42	100	100	100	100	4	1	3	1
Midazolam	7.14	326.08547	0,25-400	0.999	-0.03/-0.1	106	99	108	97	7	0	7	2
Buprenorphine	7.38	468.31081	0,25-400	1.000	0.11/0.25	101	100	100	100	3	0	3	1
Carbamazepine	7.54	237.10223	0,25-200	1.000	0.19/0.75	94	101	95	101	2	0	1	0
EDDP	7.75	278.19031	0,25-400	0.999	-0.1/-0.33	107	99	105	99	8	0	8	0
Atomoxetine	7.81	256.16958	1-200	0.999	-0.02/-0.07	109	102	104	101	4	1	7	6

Nitrazepam	8.16	282.08731	1-400	0.998	-0.09/-0.33	98	99	97	99	3	1	4	1
Oxazepam	8.22	287.05817	2-400	0.997	-0.51/-1.78	97	99	98	99	2	1	2	1
Clonazepam	8.5	316.04833	1-400	0.998	-0.35/-1.09	105	99	105	99	7	0	5	0
Lorazepam	8.6	321.0192	5-400	0.994	-0.22/-0.69	102	100	101	100	1	1	2	1
Alprazolam	8.83	309.09014	0,25-200	0.996	-0.04/-0.15	98	100	94	100	3	1	5	1
Methadone	8.89	310.21652	0,25-400	0.999	0.39/1.24	97	101	97	101	2	0	1	0
Flunitrazepam	9.19	314.09353	1-400	1.000	-0.07/-0.18	96	102	96	101	1	1	1	1
Etizolam	9.53	343.07786	0,25-200	1.000	-0.08/-0.26	105	99	105	94	2	1	2	6
Phenazepam	10.1	348.97382	2-400	0.998	-0.28/-0.82	107	99	105	99	4	0	5	1
Diazepam	10.91	285.07891	0,25-200	1.000	0.05/0.19	110	99	113	100	4	0	5	2
Diclofenac	11.4	296.02395	5-400	0.997	-0.02/-0.08	100	100	100	100	4	1	3	1
THC-OH	11.83	331.22675	5-400	0.995	-0.17/-0.56	106	99	108	97	7	0	7	2
THC-COOH	11.85	345.20602	5-400	0.992	0.17/0.5	101	100	100	100	3	0	3	1

Table S-3
Method validation in influent wastewater (n=6)

<i>Compound</i>	<i>Internal Standard</i>	<i>Matrix Suppression</i>	<i>Recovery</i>				<i>Instrumental LOQ (ng mL⁻¹)</i>	<i>Theoretical LOQ (ng L⁻¹)</i>		<i>Information</i>
			100 ng L ⁻¹	CV(%)	600 ng L ⁻¹	CV(%)				
Morphine	Atenolol-d ₇	38	73	9	68	10	0.40	4.2		
Hydroxycotinine	Atenolol-d ₇	38	120	19	88	17	0.57	5.9	*	high concentrations in "blank"
Atenolol	Atenolol-d ₇	38	92	2	91	12	0.35	3.7		
Salbutamol	Atenolol-d ₇	38	88	7	90	8	0.81	8.5		
p -Hydroxymethamphetamine	Atenolol-d ₇	38	-	-	-	-	1.34	14.0	*	high matrix suppression
Paracetamol	Atenolol-d ₇	38	-	-	-	-	1.28	13.4	*	high concentration in "blank"
Cathinone	Methcathinone-d ₃	34	~LOQ	~LOQ	51	2	5.69	67.5		
Gabapentin	Gabapentin-d ₁₀	30	-	-	-	-	3.79	51.2	*	high matrix suppression
Pseudoephedrine	Methcathinone-d ₃	34	101	5	118	5	3.10	36.7		
Methcathinone (Ephedrone)	Methcathinone-d ₃	34	85	7	94	1	0.85	10.0		
Methylone	Methylone-d ₃	37	77	2	98	3	0.04	0.4		
6-MAM	Methylone-d ₃	37	72	5	81	4	0.30	3.2		
Amphetamine	Amphetamine-d ₅	14	77	9	90	13	3.06	85.6		
MDA	MDA-d ₅	26	-	-	-	-	11.95	187.0	*	high matrix suppression
PMA	MDMA-d ₅	26	57	5	77	8	1.03	16.1		
MDMA	MDMA-d ₅	26	69	4	69	3	1.53	24.0		
Methamphetamine	Methamphetamine-d ₅	11	77	10	83	5	1.51	54.1		

Dehydronorketamine	Norketamine-d ₄	33	48	2	54	4	0.65	8.0
PMMA	MDMA-d ₅	26	74	8	79	2	0.90	14.0
Benzoylecgonine	Benzoylecgonine-d ₃	51	102	9	107	7	0.12	0.9
Mephedrone (4-MMC)	Mephedrone-d ₃	18	61	2	74	6	1.16	26.3
Norketamine	Norketamine-d ₄	33	76	5	76	11	2.89	35.5
Ketamine	Ketamine-d ₄	41	80	4	100	6	0.26	2.5
4-MEC	Ketamine-d ₄	41	73	4	91	5	0.28	2.7
Methoxetamine	Metoprolol-d ₇	40	32	7	41	3	0.19	1.9
Metoprolol	Metoprolol-d ₇	40	84	12	95	6	0.11	1.1
Methylphenidate	Metoprolol-d ₇	40	47	9	51	9	0.15	1.5
Cocaine	Cocaine-d ₃	52	93	11	103	7	0.07	0.6
Cocaethylene	Cocaine-d ₃	52	90	9	101	6	0.11	0.9
Propranolol	Fentanyl-d ₅	33	102	18	119	11	0.06	0.8
AH-7921	Fentanyl-d ₅	33	97	5	108	12	0.20	2.4
Citalopram	Buprenorphine-d ₄	24	72	14	84	10	0.09	1.5
Fentanyl	Fentanyl-d ₅	33	90	14	112	3	0.14	1.7
Midazolam	Buprenorphine-d ₄	24	98	3	119	6	0.09	1.5
Buprenorphine	Buprenorphine-d ₄	24	86	7	115	4	0.15	2.5
Carbamazepine	Buprenorphine-d ₄	24	118	6	130	6	0.14	2.3
EDDP	Atomoxetine-d ₇	23	77	16	103	11	0.11	2.0
Atomoxetine	Atomoxetine-d ₇	23	71	1	62	10	0.62	10.8
Nitrazepam	Oxazepam-d ₅	78	76	3	93	5	0.74	3.8

Clonazepam	Oxazepam-d ₅	78	57	1	46	1	1.14	5.9		
Oxazepam	Oxazepam-d ₅	78	113	15	104	14	2.23	11.5		
Lorazepam	Oxazepam-d ₅	78	86	3	87	2	2.31	11.9		
Alprazolam	Oxazepam-d ₅	78	75	15	100	14	0.16	0.8		
Methadone	Methadone-d ₃	41	97	8	96	5	0.04	0.4		
Flunitrazepam	Diazepam-d ₅	19	73	7	100	15	0.71	15.2		
Etizolam	Diazepam-d ₅	19	66	13	84	10	0.09	2.0		
Phenazepam	Diazepam-d ₅	19	104	9	118	9	1.50	32.2		
Diazepam	Diazepam-d ₅	19	125	7	110	14	0.10	2.2		
Diclofenac	Diazepam-d ₅	19	-	-	-	-	2.94	62.9	*	high matrix suppression
THC-OH	THC-OH-d ₃	19	-	-	-	-	2.41	50.3	*	high matrix suppression
THC-COOH	THC-COOH-d ₃	17	-	-	-	-	3.22	76.7	*	high matrix suppression

Table S-4
Stability of the spiked analytes (1000 ng L⁻¹) in influent WW (n=3) at pH 7. Difference (%)/ ± SD

<i>Compound</i>	Peak Area Difference (%) ± SD					
	<i>5 h</i>		<i>8 h</i>		<i>120 h</i>	
Morphine	-24	±20	-5	±17	83	±12
Hydroxycotinine	22	±5	82	±1	54	±4
Atenolol	-9	±5	-10	±4	-20	±2
Salbutamol	9	±10	1	±9	-3	±8
p-Hydroxymethamphetamine	7	±14	-1	±9	70	±22
Paracetamol	-1	±14	-8	±8	-88	±8
Gabapentin	-9	±25	-28	±8	-32	±11
Pseudoephedrine	9	±10	13	±15	0	±11
Methcathinone (Ephedrone)	-4	±18	-8	±22	-33	±11
Methylone	6	±62	-27	±21	-53	±12
6-MAM	-14	±7	-16	±8	-21	±7
Amphetamine	14	±3	23	±6	33	±7
MDA	15	±11	-2	±18	-96	±18
PMA	3	±20	16	±7	10	±7
MDMA	29	±36	6	±6	12	±6
Methamphetamine	-10	±8	-15	±33	23	±29
Dehydronorketamine	-11	±11	-7	±20	-13	±18
PMMA	-4	±33	17	±9	6	±10
Benzoylcegonine	19	±8	30	±7	56	±8
Mephedrone (4-MMC)	-1	±1	3	±1	7	±1
Norketamine	-12	±6	-24	±17	-25	±20
Ketamine	4	±27	-13	±8	-25	±5
4-MEC	5	±16	-2	±18	-32	±12
Methoxetamine	-4	±4	-6	±10	13	±0
Metoprolol	-6	±3	-9	±3	-5	±3
Methylphenidate	-10	±11	-32	±4	-88	±11
Cocaine	-9	±6	-23	±8	-75	±3
Cocaethylene	-1	±5	-6	±5	-42	±4
Propranolol	8	±6	6	±5	-27	±6
AH-7921	18	±15	9	±5	-26	±11
Citalopram	13	±9	14	±6	-23	±7
Fentanyl	9	±13	10	±2	-25	±0
Midazolam	-3	±7	5	±10	-54	±8
Buprenorphine	18	±12	23	±10	-41	±14
Carbamazepine	4	±6	4	±6	-11	±5
EDDP	45	±4	82	±5	77	±6
Atomoxetine	20	±4	22	±1	-60	±7
Nitrazepam	-14	±12	-8	±9	-79	±9
Clonazepam	-2	±11	-3	±10	-65	±9
Oxazepam	-7	±8	-1	±8	-36	±8
Lorazepam	-1	±10	-3	±7	-46	±11
Alprazolam	-1	±6	3	±7	-23	±8
Methadone	3	±6	7	±8	-41	±7
Flunitrazepam	0	±5	0	±7	-64	±4
Etizolam	-1	±5	2	±10	-41	±7
Phenazepam	2	±8	3	±15	-70	±9
Diazepam	-5	±7	-8	±10	-59	±7

Table S-5
Concentration values (ng/POCIS) for the most commonly detected drugs in the passive samplers

<i>Analyte</i>	<i>Date of collection and concentration values (ng/POCIS)</i>									
	<i>29.08.2013</i>	<i>16.09.2013</i>	<i>30.09.2013</i>	<i>15.10.2013</i>	<i>29.10.2013</i>	<i>15.11.2013</i>	<i>02.12.2013</i>	<i>18.18.2013</i>	<i>03.01.2014</i>	<i>21.01.2014</i>
Morphine	34.9	39.1	48.2	22.3	22.9	18.6	25.1	22.1	16.2	30.8
Hydroxycotinine	18.7	14.6	17.1	44.7	28.4	40.9	24.5	36.9	27.8	31.7
Atenolol	68.0	68.4	89.5	36.4	44.9	45.7	69.8	44.0	26.3	58.3
Paracetamol	718.6	521.1	940.7	778.3	779.2	699.9	1086.3	1091.3	360.5	1055.9
MDMA	33.1	33.9	48.4	25.3	25.5	4.8	49.4	32.9	20.8	26.9
Benzoylcegonine	78.0	77.8	85.6	43.1	52.7	46.1	86.0	58.9	39.4	58.9
Methoxetamine	3.3	3.5	3.8	1.5	1.9	2.0	2.6	1.4	1.2	1.8
Metoprolol	448.5	518.4	738.6	384.7	358.3	408.5	541.5	436.6	194.7	370.0
Cocaine	106.1	110.4	133.9	69.5	68.2	72.9	121.8	85.4	38.3	68.8
Propranolol	38.3	54.5	31.2	32.5	31.5	42.9	41.5	40.2	14.3	24.6
Citalopram	93.8	119.1	87.6	72.7	70.2	118.0	132.8	103.7	27.2	74.0
Carbamazepine	244.3	399.5	440.1	226.0	226.3	205.8	267.1	220.0	98.8	177.6
EDDP	97.1	169.4	139.4	103.9	81.9	91.9	114.9	78.7	20.4	74.5
Methadone	15.1	17.2	18.9	15.8	13.1	15.2	16.9	15.1	7.0	11.3
Oxazepam	696.9	927.5	744.8	692.4	525.6	675.3	754.7	745.0	187.1	421.1
Diclofenac	134.2	144.8	109.9	92.2	149.7	103.5	133.9	125.3	47.4	102.8

Table S-6
Concentration (ng/L) values of target compounds in pooled urine samples (5 mL) from anonymous Norwegian music festivals

Compound	Festival 1	Festival 2	Festival 3
Atenolol	-	900	-
Morphine	-	-	440
Paracetamol*	3.6	42.2	1320.2
Gabapentin	-	5960	-
Amphetamine	5840	-	20900
Methamphetamine	1360	820	1780
MDMA*	-	-	60.6
MDA	-	-	4340
Cocaine	-	480	520
Benzoyllecgonine	1340	78820	14680
Cocaethylene	-	-	-
Citalopram	-	-	2360
Propranolol	540	-	720
Carbamazepine	3520	7860	-
Oxazepam	340	1100	-
Methylphenidate	-	-	-
Methadone	240	-	-
THC-COOH	1020	-	7080

*($\mu\text{g/L}$)

Table S-7. Occurrence of the identified suspect candidates in the pooled urine samples by UHPLC-QTOF using the suspect database.

Compound	Number of Identified LE Ions	Retention Time	Elemental Composition	m/z
1-Benzylpiperazine (BZP)	2	1.23	C11H16N2	176.13134
Hydroxy-metoprolol	3	1.43	C15H25NO4	283.17834
Caffeine	3	2.08	C8H10N4O2	194.08036
Nadolol	3	2.27	C17H27NO4	309.19399
Metoclopramide	4	3.02	C14H22ClN3O2	299.14004
Lidocaine	2	3.19	C14H22N2O	234.1732
O-demethyl-venlafaxine	3	3.2	C16H25NO2	263.18851
Remifentanyl	3	4.37	C20H28N2O5	376.1998
Disopramide	3	4.56	C21H29N3O	339.23104
Zolpidem	4	5.19	C19H21N3O	307.16845
Venlafaxine	3	5.29	C17H27NO2	277.20416
Acetildenafil	5	5.37	C25H34N6O3	466.26922
Enalapril	2	5.49	C20H28N2O5	376.1998
Mepyramine	2	5.52	C17H23N3O	285.1841
Sildenafil	2	6.19	C22H30N6O4S	474.2049
Benzocaine	2	6.5	C9H11NO2	165.07897
Imipramine	2	7.67	C19H24N2	280.19393
Cetirizine	2	8.28	C21H25ClN2O3	388.15535
Trimipramine	2	8.44	C20H26N2	294.20958
Irbesartan	2	9.08	C25H28N6O	428.23244
Irbesartan	2	9.28	C25H28N6O	428.23244
Cinnarizine	2	10.48	C26H28N2	368.22523
Fluocinonide	2	11.17	C26H32F2O7	494.21159
Orlistat	4	13.24	C29H53NO5	495.39234

(1) Harman, C.; Reid, M.; Thomas, K. V. *Environ Sci Technol* **2011**, *45*, 5676-5682.