

Accepted Manuscript

This is an Accepted Manuscript of the following article:

J.A. Baz-Lomba, Arndís Sue-Ching Löve, Malcolm J. Reid, Kristín Ólafsdóttir, Kevin V. Thomas. A high-throughput solid-phase microextraction and post-loop mixing large volume injection method for water samples. *Journal of Chromatography A*. Volume 1531, 2018, pages 32-38, ISSN 0021-9673.

The article has been published in final form by Elsevier at

<http://dx.doi.org/10.1016/j.chroma.2017.11.051>

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1 A high-throughput solid-phase microextraction and post-loop mixing large 2 volume injection method for water samples.

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8

9 **Abstract:**

10 This article presents a novel approach for the analysis of 13 drugs in wastewater for use in
11 wastewater-based epidemiology (WBE) studies. Sample preparation remains one of the
12 principal bottlenecks in modern high-throughput analysis by ultra-high-performance liquid
13 chromatography-tandem mass spectrometry (UHPLC-MS/MS). The proposed methodology is
14 based on the micro-extraction of small volumes (1 mL) of wastewater using a HLB 96-well
15 microplate and both large volume injection (LVI) and post-loop mixing injection (PLM). With
16 this configuration, the limits of quantification (LOQ) were below the reported environmental
17 concentrations of the target compounds in wastewater. Furthermore, both the complexity of
18 collecting, transporting and storing the wastewater sample, sample preparation time, cost and
19 amount of solvent used are all diminished, enhancing the suitability of this methodology for
20 future WBE studies. A new workflow is also proposed in order to create a virtual specimen
21 library bank for WBE by using high-resolution mass spectrometry (HRMS). The method was
22 validated and the limits of quantification were between 0.2 and 6.3 ng L⁻¹. The relative standard
23 deviations (RSD) for a standard mixture at 200 ng L⁻¹ (n=6) was between 3.4 and 14.4% while
24 the recoveries for the 13 drug target residues (DTR) were between 92 and 110%. The developed
25 and validated method was finally successfully applied to 10 wastewater samples collected from
26 Oslo, Norway.

27 **Keywords**

28 Wastewater-based epidemiology, Liquid chromatography-mass spectrometry, High-
29 throughput analysis, 96-well plate microextraction, Post-loop mixing, Large volume injection

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1. Introduction

38 Wastewater-based epidemiology (WBE) has been established as a complementary tool to
39 estimate drug use at the population level by the quantitative measurement of endogenous and
40 exogenous biomarkers excreted by humans in wastewater [1]. Recently WBE has also been
41 shown to be an effective approach for estimating population level human exposure to a wide
42 range of pollutants [2, 3]. WBE has the potential to provide real-time data on geographical and
43 temporal trends in illicit drug use [4]. Traditional methods used for this purpose are usually
44 based on population surveys, sales data, clinical cases, seizures or mortality rates related to use,
45 but these approaches lack representativeness, are time consuming and expensive [5].

46 The WBE procedure consists of several steps involving sample collection, chemical analysis
47 and the drug target residue (DTR) back-calculation, which are subject to a certain number of
48 sources of uncertainty that have been described and progressively diminished by using a
49 harmonized approach [6]. The appropriate collection of representative composite wastewater
50 samples to compensate for the flow fluctuations during the sampling has been described by Ort
51 and colleagues [7], presenting an acceptable uncertainty when estimating the population
52 weighted loads of around 5 to 10% [6]. Furthermore, wastewater data has been shown to present
53 low temporal representativeness when assessing annual averages [8]. Consequently, the annual
54 estimates for a certain substance based on WBE studies must consist of several stratified
55 random samples (typically 56 samples per year for an acceptable level of sample size related
56 uncertainty < 10% [9]) rather than only one consecutive week as most of the WBE studies, such
57 as the European-wide monitoring for the European Monitoring Centre for Drugs and Drug
58 Addiction (EMCDDA) [10]. However, increasing the sampling frequency to decrease the
59 annual estimate uncertainty may therefore imply a greater activity from the wastewater
60 treatment plant (WWTP) operators in order to collect the samples to be analyzed. Therefore,
61 there is a need to develop more suitable and cost-effective alternatives to classic methods for
62 the long-term monitoring of exposure and substance use at community level through WBE [8].

63 Sample analysis is critical to achieve reliable concentration of the DTR. The uncertainty related
64 to the analytical variability is estimated to be up to 26% [6]. Most of the DTR are found in
65 wastewater in the ng L^{-1} range and therefore a pre-concentration step is usually required [11].
66 Solid phase extraction (SPE) is the most common procedure for this purpose and large volumes
67 of wastewater are necessary in order to reach the required limits of detection for determining
68 environmental concentrations (between 50 and 1000 mL) [12]. However, the majority of the
69 above procedures are tedious and time-consuming. Miniaturization of the sample preparation
70 has become an alternative in modern high-throughput methods. Solid phase microextraction
71 (SPME) differs from SPE in the ratio sorbent *versus* sample volume. Therefore, all the different
72 SPME configurations are an equilibrium extraction technique since only a small portion of the
73 analyte is extracted from the sample whereas SPE techniques are based on the complete
74 extraction of all the analytes from the sample. Micro-SPE (μSPE) is a miniaturized version of
75 SPE with the same concept of extracting all the analytes but in this case, with a smaller sample
76 volume and a reduced amount of packed sorbent [13].

77

78 Large volume injection (LVI) methods are another alternative that provide the advantage of
79 reducing sample preparation steps, improving the reproducibility and minimizing potential
80 contamination of the sample. Furthermore, LVI increases sample throughput at minimal cost
81 [14] and the water sample can be injected in the initial aqueous mobile phase without causing
82 serious peak broadening. However, to date, LVI methods have normally presented low
83 sensitivity with respect to the environmental levels [15], and require modern and very sensitive
84 instruments that are not always available in the analytical laboratories [16].

85 Ultra-high-performance liquid chromatography (UHPLC) has recently emerged providing
86 higher sensitivity, better separations and improved throughput [5]. UHPLC columns are packed
87 with much smaller particles and support greater pressures that increases the efficiency and
88 decreases the run time. However, UHPLC columns become a problem when using LVI due to
89 lower sample capacity leading to chromatographic distortions such as peak broadening or
90 volume over-load issues [17]. The post-loop mixing (PLM) approach efficiently avoids the
91 above problems by completely diluting the sample into organic mobile phase before the sample
92 reaches the mixer and is diluted and carried to the column by the aqueous mobile phase. The
93 initial elution solvent rate is such that the sample is retained at the head of the column in a
94 narrow band (i.e. A:water 97%; B:methanol 3%). In this case, rather than injecting the
95 wastewater sample directly, the sample is extracted by μ SPE and then a larger volume of the
96 eluent is injected into the system directly in organic solvent without reconstitution in water.

97 At present the main development focus within the WBE field is based on the development of
98 analytical methods for new markers [18-20] and reduction of the uncertainty related to both the
99 in-sewer transformation [21] and the estimation of the population of the WWTP catchment
100 areas [22]. However, due to the relatively low uncertainty and the inter-laboratory exercises for
101 external quality control assurance, the analytical methods have remained unaltered, tedious and
102 inefficient. Therefore, the combination of μ SPE with PLM together with LVI provides a perfect
103 compromise between sample throughput, cost, sensitivity and chromatographic separation.

104 The aim of this study was to develop, validate and apply a novel high-throughput WBE
105 procedure for the analysis of 13 DTR by off-line μ SPE-PLM-LVI-UHPLC coupled to tandem
106 mass spectrometry (MS/MS). The selected compounds were amphetamine, methamphetamine,
107 3,4-methylenedioxymethamphetamine (MDMA), benzoylecgonine, cocaine, cocaethylene,
108 atenolol, citalopram, carbamazepine, fexofenadine, methylphenidate, metoprolol and lidocaine.
109 Thus, this procedure will potentially improve the technical and environmental WBE feasibility
110 by: i) reducing sample preparation and analysis time; ii) reducing costs; iii) reducing the amount
111 of solvents needed; iv) improving the whole method efficiency, v) making the sample collection
112 and storage easier for the WWTP operator (from 1L to 5 mL or from one big bottle to one small
113 glass vial) and vi) enabling the creation of a virtual specimen library bank for WBE by archiving
114 and retrospectively analyzing the data acquired in HRMS mode. Finally, to demonstrate the
115 feasibility of this approach, μ SPE-PLM-LVI-UHPLC-MS/MS was applied to the analysis of
116 10 wastewater samples.

117

118 2. Experimental

119 2.1 Reagents and materials.

120 Reference standards for 13 drugs and/or their main metabolites chosen for the analysis were the
121 following: amphetamine, methamphetamine, MDMA, cocaine, benzoylecgonine,
122 cocaethylene, atenolol, citalopram, carbamazepine, fexofenadine, methylphenidate,
123 metoprolol, and lidocaine dissolved in methanol (MeOH) or acetonitrile (ACN) at
124 concentrations of 1 mg mL^{-1} or $100 \text{ } \mu\text{g mL}^{-1}$. Standard solutions of each compound were made
125 in methanol at $100 \text{ } \mu\text{g mL}^{-1}$ and then diluted into final mix solutions to a concentration of 10
126 and 1 ng mL^{-1} . Corresponding isotope-labeled internal standards (ILIS) were amphetamine-d8,
127 methamphetamine-d11, MDMA-d5, cocaine-d3, benzoylecgonine-d3, cocaethylene-d3,
128 atenolol-d7, fexofenadine-d6, metoprolol-d7 and lidocaine-d6 dissolved in MeOH or ACN at
129 concentrations of $100 \text{ } \mu\text{g mL}^{-1}$. The ILIS solutions were made in methanol at $10 \text{ } \mu\text{g mL}^{-1}$ and
130 then diluted to a mix working solution at 10 ng mL^{-1} . All reference standards and ILIS were
131 purchased from Cerilliant (Round Rock, TX, USA). The standards and working solutions were
132 stored at $-20 \text{ } ^\circ\text{C}$.

133 HPLC-grade MeOH was purchased from Rathburn Chemicals Ltd. (Walkerburn, SCT, UK).
134 HPLC-grade ACN was acquired from VWR Chemicals (Oslo, Norway). Ammonium hydroxide
135 (NH_4OH) solution $\geq 25\%$ in water was obtained from Fluka - Sigma-Aldrich (Oslo, Norway)
136 and formic acid (FA) 98-100% (for analysis) was purchased from Merck - Millipore (Oslo,
137 Norway).

138 2.2 Wastewater samples

139 Influent wastewater samples were collected from Vestfjorden Avløpselskap (VEAS), the Oslo
140 wastewater treatment plant (WWTP) in June 2016. A total of 10 flow proportional samples
141 were collected with an EFCON® Wall Mounted Vacuum sampler from the VEAS raw inlets
142 between the 17th and the 30th of June. The sampler was operated at $4 \text{ } ^\circ\text{C}$ and the wastewater
143 samples were firstly collected in high-density polyethylene (HDPE) bottles and then
144 homogenized, poured into the 7 mL glass vials and stored at $-20 \text{ } ^\circ\text{C}$ immediately following
145 collection.

146 Weekend composite samples consisted of a three-day composite sample from Friday (08:00) to
147 Monday (08:00) while weekdays were twenty-four-hour composite samples. VEAS treats
148 sewage for a *de jour* population of approximately 600,000 people of which the city contributes
149 about 70.5% and the adjoining areas representing the other 29.5%. The total length of the sewer
150 line is 42.3 km and the mean residence time in the sewer system is 5 hours [23].

151

152 2.3 Sample preparation and μ -SPE

153 Sample preparation is a crucial step to remove any matrix components that may compete with
154 the target analytes in the ionization process during the UHPLC-MS/MS analysis. Prior to
155 extraction, 5 mL of influent wastewater were spiked with 50 μ L of the ILIS working solution
156 to reach a concentration of 100 ng L⁻¹. Following vortex stirring, 1 ml of sample was centrifuged
157 at 16,200 \times g for 5 min at 4°C in a Heraeus Fresco Biofuge (Thermo Scientific, Waltham, MA,
158 USA) and the supernatant was used for analysis. μ SPE was performed using Waters Oasis HLB
159 μ Elution plates, 30 μ m (Milford, MA, USA). The plate was conditioned by washing and rinsing
160 with 1 ml of MeOH and 1 ml of ultrapure water under suction. The wastewater samples were
161 loaded onto the plate under suction and washed with 1 ml of ultrapure water. The plate was
162 vacuum dried for 15 min. Analytes were eluted into a 96 well plate using 50 μ l of 1% NH₄OH
163 in MeOH, 100 μ l of MeOH and 50 μ l of 1% FA in MeOH.

164 The final 200 μ l extract was divided in two LC vials for separate analysis for both target and
165 retrospective purposes (Figure 1). No solvent evaporation or residue re-dissolution were needed
166 before injection and therefore, the eluent consisted only of methanol. Analysis was performed
167 by injecting 37 μ l into the PLM-LVI-UHPLC-MS/MS.

168 2.4 LC–MS/MS analysis

169 Wastewater analysis was carried out with a Waters Acquity UPLC system (Milford, MA, USA)
170 equipped with a binary solvent manager and a sample manager. The UHPLC was coupled to a
171 Waters Quattro Premier XE Micromass triple quadrupole mass spectrometer (Milford, MA,
172 USA) with a T-wave collision cell and electrospray ionization interface (ESI), operated in
173 positive ionization mode. Selected parent and product ions together with ionization and
174 collision energy parameters are presented in Table 1. Mass spectrometer parameters were tuned
175 with a direct infusion of standard solutions. Information about the HRMS acquisition
176 parameters and other information can be found in Baz-Lomba et al. 2016 [24].

177 Chromatographic separation was carried out using a Waters Acquity UPLC BEH C8 column,
178 1.7 μ m, 2.1 x 100 mm (Milford, MA, USA). The column temperature was kept at 50°C and the
179 temperature of the sample manager was 4°C. A constant flow rate of 0.4 ml min⁻¹ was used
180 with a mobile phase consisting of 0.1% ammonium hydroxide (solvent A) and acetonitrile
181 (solvent B). The elution gradient changed as follows: 0 min (3% B); 4.9 min (3% B); 5.1 min
182 (40% B); 8.5 min (60% B); 9 min (95% B); 10 min (95% B); 10.5 min (3% B); 11 min (3% B).
183 The sample injection volume was 37 μ L.

184 The cone and desolvation gas used was nitrogen with flow rates of 50 L h⁻¹ and 800 L h⁻¹,
185 respectively. The collision gas used was argon with a flow rate of 0.15 mL min⁻¹. Other
186 operational parameters were capillary voltage, 3.2 kV; source temperature, 100 °C and
187 desolvation temperature, 450 °C. The loop and needle volumes were 50 and 250 μ L respectively
188 and the injection mode was partial loop with needle overfill mode (PLNO). The PLNO mode
189 provides the best partial loop accuracy, precision, and linearity and only sample and mobile
190 phase were injected onto the column avoiding air gaps or weak wash solvent.

191 Data acquisition was performed working in multiple reaction-monitoring mode (MRM).
192 Infusion solutions of individual standards were prepared to optimize MS conditions and to
193 select MS/MS transitions for both target analytes and ILIS. The best results in terms of

194 sensitivity were those using ESI operating in positive ionization mode, using the protonated
195 molecule $[M+H]^+$ as precursor ion. The most abundant product ion of each target analyte was
196 typically used for quantification and one additional product ion was used for confirmation.
197 Furthermore, the retention times were also compared with those from reference standards (\pm
198 0.2 minutes). Each DTR was quantified using its ILIS as a surrogate internal standard, except
199 citalopram, carbamazepine and methylphenidate for which the ILIS with the most similar
200 retention time and chemical structures were selected. All data were acquired and processed
201 using MassLynx v4.1 (Milford, MA, USA).

202 2.5 Method validation

203 Method validation was performed in terms of linearity, method quantification limits (LOQ),
204 relative and absolute recoveries (trueness), repeatability and matrix effects. The performance
205 of the method was evaluated following EU guidelines with minor modifications [25]. The
206 linearity of the method was studied by analyzing standard solutions in methanol in triplicate at
207 eight concentrations, in the range of 0.025 to 10 ng mL⁻¹, together with the ILIS at 0.5 ng mL⁻¹.
208 Satisfactory linearity was considered when the correlation coefficient (R^2) was higher than
209 0.99, based on relative responses (analyte peak area/ILIS peak area). The LOQs were calculated
210 in wastewater samples with known concentrations (all compounds were present in sample) as
211 the concentrations giving a signal-to-noise ratio (S/N) of ≥ 10 .

212 Relative and absolute recoveries were tested in triplicate in wastewater samples spiked at 100
213 ng L⁻¹. Adequate blank samples were not found since the target compounds were present in all
214 the wastewater samples. Therefore, an additional set of three wastewater samples were analyzed
215 by spiking only the ILIS before extraction to account for the analyte background. Relative
216 recoveries were calculated by spiking the ILIS before the μ SPE while for the absolute
217 recoveries, meant for the assessment of the μ SPE efficiency, were spiked after the extraction,
218 right before the injection in the LC-MS/MS system. Calibration standards in solvent were used
219 for quantification and the relative recoveries between 80% and 120% were considered
220 satisfactory. Precision (expressed as repeatability) was assessed as the relative standard
221 deviation (RSD) of six wastewater samples spiked at 200 ng L⁻¹. The matrix effects that
222 occurred during the ionization (ESI) were assessed by spiking three wastewater extracts at 1 ng
223 mL⁻¹ together with the respective ILIS right before analysis and comparing its responses with
224 that for those spiked at the same concentration in mobile phase. A non-spiked wastewater
225 sample (only with ILIS) was analyzed simultaneously to subtract its response from the spiked
226 sample:

$$227 \quad \text{Matrix effect (\%)} = \frac{\text{Response in ww extract} - \text{Response ww blank}}{\text{Response in mobile phase}} \times 100$$

228

229 3. Results and discussion

230 3.1 Large volume injection and post-loop mixing injection

231 The SPE extract is commonly evaporated under a current of nitrogen and reconstituted into the
232 initial mobile phase to improve the chromatographic separation and avoid the sample to
233 significantly penetrate the column without an optimal retention [24]. The PLM configuration,
234 described in Figure 2, avoids the eluent reconstitution following μ SPE and chromatographic
235 peak distortion when using LVI with UHPLC. In the PLM-LVI configuration, the position of
236 the mixer and line A (aqueous phase) are changed in such a way that line B (organic phase,
237 acetonitrile) goes directly to the loop in the autosampler, drags the sample and meets the
238 aqueous phase in the mixer located right after the autosampler and before the HPLC column.
239 At this stage, the sample is diluted in the mixer and stacked at the head of the column.
240 Furthermore, the PLM-LVI configuration mitigates one of the main issues when using LVI with
241 UHPLC columns related to the lower sample capacity leading into chromatographic distortions
242 such as peak broadening or volume over-load issues. By using a high initial water ratio (i.e
243 97%), the sample is completely diluted in water right before the UHPLC column and retained
244 in a narrow band at the head of the column.

245 The ratio of the organic phase versus aqueous phase will depend on the characteristics of the
246 target compounds and becomes a critical feature in the development of the method.
247 Optimization of the percentages of organic phase (acetonitrile) in water was achieved by
248 comparing the peak shapes of the early-eluting compounds. The initial gradient was tested at
249 1,2,3,4,5 and 10% of acetonitrile. If the initial ratio of acetonitrile was too high, the polar
250 analytes could not be retained at the column head due to the strong elution strength and
251 therefore, the peak width of the analytes increased significantly. Both the loop and tubing (from
252 autosampler to mixer) volumes were taken into account to estimate the time to fill the loop and
253 drag the sample into the column (approximately 60 μ L). The best compromise between peak
254 shape and total run time was found to be 3% acetonitrile in water. Using a flow rate of 0.4 mL
255 min^{-1} , the initial gradient was held for 5 minutes at 3% acetonitrile and once the analytes were
256 retained at the head of the column the % acetonitrile was increased.

257 3.2 Method validation

258 The principal aim of this study was to prove the concept and applicability of a μ SPE- PLM-
259 LVI -UHPLC-MS/MS configuration for WBE. Therefore, neither the μ SPE nor the UHPLC
260 conditions were optimized. However, all the conditions and parameters used in this study were
261 previously developed “in-house” for validated and published methods [24, 26]. Furthermore,
262 the analytical method used in this study has been validated through an external inter-laboratory
263 exercise with other 27 international laboratories for some of the studied compounds (cocaine,
264 benzoylecgonine, amphetamine, methamphetamine and MDMA), successfully meeting all the
265 external quality control requirements [27].

266 The mean correlation coefficients (R^2) of the calibration curves, which are higher than 0.99
267 (Table 2) show good linearity of the method in the range of 0.025 to 10 ng mL^{-1} . The method
268 LOQs were below 10 ng L^{-1} for all the compounds, ranging from 0.2 ng L^{-1} for carbamazepine
269 to 6.3 ng L^{-1} for MDMA, being better than achieved with and SPE-UHPLC-MS/MS method on
270 the same 16-year old MS system [26] and were below the reported environmental
271 concentrations of the target compounds in wastewater.

272

273 The absolute recoveries for the μ SPE performed with Waters Oasis HLB were satisfactory with
274 values higher than 79% for all the compounds except for amphetamine with only a 36%
275 recovery. Satisfactory relative recoveries were found for all the compounds, ranging from 92%
276 for citalopram to 110% for cocaine. Precision ($n=6$) for spiked wastewater samples at 200 ng
277 L^{-1} was satisfactory in all cases with RSD values ranged from 3.4 to 14.4%.

278 3.3 Matrix effects

279 Ion suppression or enhancement is commonly observed in complex environmental matrices
280 such as wastewater as a consequence of the matrix effect, which affects sensitivity, accuracy
281 and the evaluation of method recovery. The matrix effect observed for the target compounds
282 dissolved in wastewater is presented in Table 2. Little or no signal suppression was observed
283 for MDMA, citalopram, carbamazepine and metoprolol. Atenolol and fexofenadine, both co-
284 eluting at the beginning of the chromatographic run, showed a high ion suppression while the
285 rest of the compounds showed a moderate ions suppression/enhancement ($\pm 20\%$). The matrix
286 suppression and recoveries were acceptable for the compounds for which no corresponding
287 isotope-labelled internal standards were available.

288 3.4 Analysis of wastewater samples

289 The developed method was applied to the analysis of ten 24-hour flow proportional influent
290 samples (72-hour for the weekend samples). Standard calibration curves were used to calculate
291 the concentrations of the target compounds and injected in duplicate at the beginning and at the
292 end of the run. Fortified "blank" samples were injected as internal quality control during the
293 sequence.

294 The target compounds were found in all the inlet wastewater samples with changing
295 concentrations (Table 3). Carbamazepine showed the highest concentrations with an average
296 ($n=10$) of 1200 ng L^{-1} while cocaethylene and metoprolol showed the lowest concentrations
297 with an average of 9 and 7 ng L^{-1} respectively. Amphetamine and methamphetamine
298 concentrations show similar levels ranging from 200 to 600 ng L^{-1} respectively. MDMA was
299 the compound with the highest coefficient of variance among the 10 samples (61%) when
300 comparing week days with the weekend due to its recreational use during the weekend in
301 agreement with previous works [28]. Cocaine and its main metabolite, benzoylecgonine, ranged
302 from 100 to 700 ng L^{-1} and show a benzoylecgonine/cocaine ratio of approximately 2~3, in
303 agreement with previous publications [12]. For the rest of the pharmaceuticals, concentrations
304 ranged from 25 to 48 ng L^{-1} for atenolol, from 35 to 71 ng L^{-1} for citalopram, from 117 to 205
305 ng L^{-1} for fexofenadine, from 140 to 263 ng L^{-1} for methylphenidate and from 55 to 108 ng L^{-1}
306 for lidocaine.

307 3.5 Environmental feasibilities and implications for the future

308 In summary, the 96-well plate for μ SPE provides the highest throughput for the analysis of
309 wastewater samples to date. The main advantages are the reduction of the time invested per
310 sample, the final cost per sample is lower (only the cartridges are approximately 25% less
311 expensive and the amount of ILIS used compared with classic methods is approximately 100
312 times less), slightly decrease of matrix effects due to the reduction of the volume extracted and
313 from the environmental point of view, is more feasible due to the reduction of the solvents used
314 for the extraction, by approximately a 90%.

315 Furthermore, the HLB sorbent, with a hydrophilic-lipophilic-balanced sorbent, offers the
316 possibility to extract a wide range of compounds with different psychochemical characteristics
317 enabling the simultaneous analysis of a wide range of drugs and pharmaceuticals in one single
318 extraction. The use of this generic extraction methodology also is very suitable for HRMS and
319 retrospective analysis, which have been proposed as a good alternative for data storage and
320 environmental repository without the need of additional sample analyses [29]. Furthermore,
321 this workflow does not imply additional extractions and both analysis are performed using the
322 same extract. Therefore, the approach proposed in Figure 1 will allow the performance of
323 different tasks such as pre- and post-target analysis, potential elucidation of metabolites and
324 transformation products, retrospective analysis and non-target analysis only with one extraction
325 and two analysis.

326 The PLM-LVI configuration complements the μ SPE reducing even more the sample
327 preparation time by avoiding the reconstitution of the eluent. Furthermore, this configuration
328 also improves the efficiency of the method by injecting larger volumes. Most of the published
329 analytical methods for the analysis of wastewater samples reconstitute the eluent in 250-1000
330 μ L for a final injection of a few μ L (normally between 2-5 μ L) [24, 30]. In this study, we elute
331 200 μ L that are split in two for target and retrospective analysis, and 37 μ L out of 100 μ L are
332 injected into the system. This configuration would also allow the introduction of robots or
333 automated μ SPE that would simplify and improve substantially the method in the future.

334 The reduction of the time invested for collecting, extracting and analyzing the sample together
335 with substantial reduction of the cost, increase the possibilities for the laboratories to perform
336 real-time monitoring. The fact that the staff at the WWTP move from collecting 500-1000 ml
337 in big plastic bottles to 5 mL in small glass vials could potentially increase the number of
338 collaborations between laboratories and treatment plants.

339 4. Conclusions

340 A novel analytical methodology based on the use of μ SPE-PLM-LVI-UHPLC-MS/MS has
341 been developed for the simultaneous quantification and confirmation of 13 widely consumed
342 drugs in urban wastewater and applied to 10 influent wastewater samples from Oslo, Norway.
343 A high throughput analytical procedure has been fully validated, obtaining satisfactory accuracy
344 and precision and high sensitivity. The method LOQs are comparable with previous studies and
345 below the environmental concentrations found in Oslo during the last years.

346 The combination of μ SPE with PLM-LVI has been demonstrated to be a promising compromise
347 to reduce the sample preparation time and still reach the required detection levels for
348 environmental samples. Furthermore, reducing total cost and amounts of solvents, increasing
349 the method efficiency and improving the collection and handling of the samples, have upgraded
350 the technical and environmental feasibility of classic WBE methods. These results highlight the
351 potential of μ SPE-PLM-LVI-UHPLC-MS/MS for WBE studies in the future.

352 In addition, a 200 μ L μ SPE extract is enough for both the quantitative and HRMS analysis,
353 which will enable the creation of a virtual specimen library bank for WBE. This additional
354 workflow will archive all the data for retrospective analysis, functioning as a backup for cases
355 when old samples are not available or degraded.

356

357 **Acknowledgments**

358 This work was supported by the COST Action ES1307 “SCORE – Sewage biomarker analysis
359 for community health assessment”, from the European Cooperation in Science and Technology
360 framework. The authors thank Pia Ryrfors, VEAS WWTP, for providing access to the sampling
361 area and the best information regarding the wastewater samples.

362

363 [1] K.V. Thomas, L. Bijlsma, S. Castiglioni, A. Covaci, E. Emke, R. Grabic, F. Hernández, S. Karolak, B.
364 Kasprzyk-Hordern, R.H. Lindberg, Comparing illicit drug use in 19 European cities through sewage
365 analysis, *Sci Total Environ*, 432 (2012).

366 [2] Y. Ryu, E. Gracia-Lor, R. Bade, J. Baz-Lomba, J.G. Bramness, S. Castiglioni, E. Castrignanò, A.
367 Causanilles, A. Covaci, P. de Voogt, Increased levels of the oxidative stress biomarker 8-iso-
368 prostaglandin F₂α in wastewater associated with tobacco use, *Scientific Reports*, 6 (2016).

369 [3] I. González-Mariño, R. Rodil, I. Barrio, R. Cela, J.B. Quintana, Wastewater-Based Epidemiology as a
370 New Tool for Estimating Population Exposure to Phthalate Plasticizers, *Environmental Science &
371 Technology*, 51 (2017) 3902-3910.

372 [4] S. Castiglioni, K.V. Thomas, B. Kasprzyk-Hordern, L. Vandam, P. Griffiths, Testing wastewater to
373 detect illicit drugs: state of the art, potential and research needs, *Sci Total Environ*, 487 (2014).

374 [5] J.A. Baz-Lomba, M.J. Reid, K.V. Thomas, Target and suspect screening of psychoactive substances
375 in sewage-based samples by UHPLC-QTOF, *Analytica Chimica Acta*, 914 (2016) 81-90.

376 [6] S. Castiglioni, L. Bijlsma, A. Covaci, E. Emke, F. Hernández, M. Reid, C. Ort, K.V. Thomas, A.L.N. van
377 Nuijs, P. de Voogt, E. Zuccato, Evaluation of Uncertainties Associated with the Determination of
378 Community Drug Use through the Measurement of Sewage Drug Biomarkers, *Environmental Science
379 & Technology*, 47 (2013) 1452-1460.

380 [7] C. Ort, M.G. Lawrence, J. Rieckermann, A. Joss, Sampling for pharmaceuticals and personal care
381 products (PPCPs) and illicit drugs in wastewater systems: are your conclusions valid? A critical review,
382 *Environ Sci Technol*, 44 (2010).

383 [8] J.A. Baz-Lomba, C. Harman, M. Reid, K.V. Thomas, Passive sampling of wastewater as a tool for the
384 long-term monitoring of community exposure: Illicit and prescription drug trends as a proof of concept,
385 *Water Research*, 121 (2017) 221-230.

386 [9] C. Ort, J.M. Eppler, A. Scheidegger, J. Rieckermann, M. Kinzig, F. Sörgel, Challenges of surveying
387 wastewater drug loads of small populations and generalizable aspects on optimizing monitoring
388 design, *Addiction*, 109 (2014) 472-481.

389 [10] C. Ort, A.L. Nuijs, J.D. Berset, L. Bijlsma, S. Castiglioni, A. Covaci, P. Voogt, E. Emke, D. Fatta-
390 Kassinos, P. Griffiths, Spatial differences and temporal changes in illicit drug use in Europe quantified
391 by wastewater analysis, *Addiction*, 109 (2014).

392 [11] P. Vazquez-Roig, C. Blasco, Y. Pico, Advances in the analysis of legal and illegal drugs in the aquatic
393 environment, *Trac-Trend Anal Chem*, 50 (2013) 65-77.

394 [12] A.L. van Nuijs, S. Castiglioni, I. Tarcomnicu, C. Postigo, M. Lopez de Alda, H. Neels, E. Zuccato, D.
395 Barcelo, A. Covaci, Illicit drug consumption estimations derived from wastewater analysis: a critical
396 review, *Sci Total Environ*, 409 (2011) 3564-3577.

397 [13] D. Vuckovic, High-throughput solid-phase microextraction in multi-well-plate format, *Trac-Trend
398 Anal Chem*, 45 (2013) 136-153.

399 [14] C. Boix, M. Ibanez, J.V. Sancho, J. Rambla, J.L. Aranda, S. Ballester, F. Hernandez, Fast
400 determination of 40 drugs in water using large volume direct injection liquid chromatography-tandem
401 mass spectrometry, *Talanta*, 131 (2015) 719-727.

402 [15] M.J. Bueno, S. Ucles, M.D. Hernando, E. Davoli, A.R. Fernandez-Alba, Evaluation of selected
403 ubiquitous contaminants in the aquatic environment and their transformation products. A pilot study
404 of their removal from a sewage treatment plant, *Water Res*, 45 (2011).

405 [16] M. Wu, Y. Qian, J.M. Boyd, S.E. Hrudey, X.C. Le, X.F. Li, Direct large volume injection ultra-high
406 performance liquid chromatography-tandem mass spectrometry determination of artificial
407 sweeteners sucralose and acesulfame in well water, *J Chromatogr A*, 1359 (2014) 156-161.

408 [17] Q. Zhong, L. Shen, J. Liu, D. Yu, S. Li, J. Yao, S. Zhan, T. Huang, Y. Hashi, S. Kawano, Z. Liu, T. Zhou,
409 Pre-column dilution large volume injection ultra-high performance liquid chromatography-tandem
410 mass spectrometry for the analysis of multi-class pesticides in cabbages, *J Chromatogr A*, 1442 (2016)
411 53-61.

412 [18] H. Rapp-Wright, G. McEneff, B. Murphy, S. Gamble, R. Morgan, M. Beardah, L. Barron, Suspect
413 screening and quantification of trace organic explosives in wastewater using solid phase extraction and

414 liquid chromatography-high resolution accurate mass spectrometry, *Journal of Hazardous Materials*,
415 329 (2017) 11-21.

416 [19] Y. Ryu, M.J. Reid, K.V. Thomas, Liquid chromatography–high resolution mass spectrometry with
417 immunoaffinity clean-up for the determination of the oxidative stress biomarker 8-iso-prostaglandin
418 F2alpha in wastewater, *Journal of Chromatography A*, 1409 (2015) 146-151.

419 [20] I. Gonzalez-Marino, E. Gracia-Lor, N.I. Rousis, E. Castrignano, K.V. Thomas, J.B. Quintana, B.
420 Kasprzyk-Hordern, E. Zuccato, S. Castiglioni, Wastewater-Based Epidemiology To Monitor Synthetic
421 Cathinones Use in Different European Countries, *Environ Sci Technol*, 50 (2016) 10089-10096.

422 [21] A.K. McCall, R. Palmitessa, F. Blumensaat, E. Morgenroth, C. Ort, Modeling in-sewer
423 transformations at catchment scale - implications on drug consumption estimates in wastewater-
424 based epidemiology, *Water Res*, 122 (2017) 655-668.

425 [22] K.V. Thomas, A. Amador, J.A. Baz-Lomba, M. Reid, Use of Mobile Device Data To Better Estimate
426 Dynamic Population Size for Wastewater-Based Epidemiology, *Environmental Science & Technology*,
427 51 (2017) 11363-11370.

428 [23] V.A. (VEAS), Annual report 2016, in: <http://www.veas.nu/home/om-veas/%C3%A5rsmeldinger>
429 (Ed.), Oslo. Norway, 2017.

430 [24] J.A. Baz-Lomba, M.J. Reid, K.V. Thomas, Target and suspect screening of psychoactive substances
431 in sewage-based samples by UHPLC-QTOF, *Anal Chim Acta*, 914 (2016) 81-90.

432 [25] B. Magnusson, The fitness for purpose of analytical methods: A laboratory guide to method
433 validation and related topics (2014), Eurachem, 2014.

434 [26] M.J. Reid, K.H. Langford, J. Mørland, K.V. Thomas, Quantitative assessment of time dependent
435 drug-use trends by the analysis of drugs and related metabolites in raw sewage, *Drug Alcohol Depend*,
436 119 (2011).

437 [27] SCORE, Interlaboratorium Tests (2011–2017), in: Sewage Analysis Core Group Europe, SCORE,
438 2017.

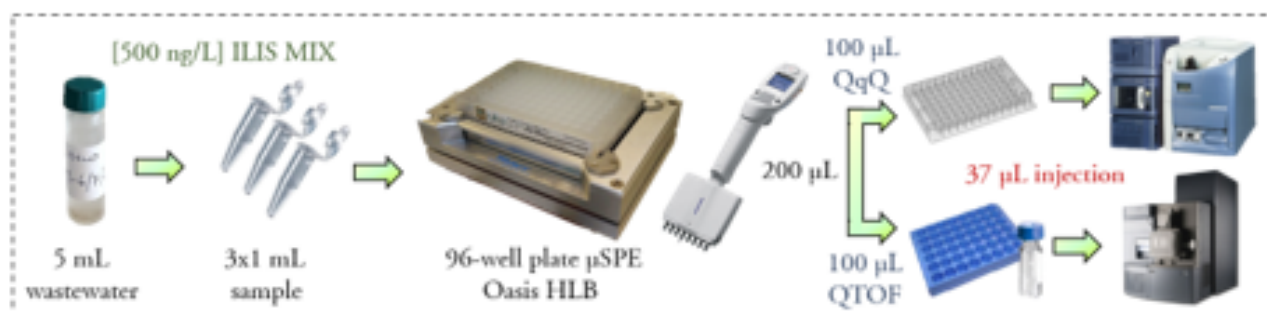
439 [28] J.A. Baz-Lomba, S. Salvatore, E. Gracia-Lor, R. Bade, S. Castiglioni, E. Castrignano, A. Causanilles, F.
440 Hernandez, B. Kasprzyk-Hordern, J. Kinyua, A.K. McCall, A. van Nuijs, C. Ort, B.G. Plosz, P. Ramin, M.
441 Reid, N.I. Rousis, Y. Ryu, P. de Voogt, J. Bramness, K. Thomas, Comparison of pharmaceutical, illicit
442 drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for
443 8 European cities, *BMC Public Health*, 16 (2016) 1035.

444 [29] E.L. Schymanski, H.P. Singer, J. Slobodnik, I.M. Ipolyi, P. Oswald, M. Krauss, T. Schulze, P. Haglund,
445 T. Letzel, S. Grosse, N.S. Thomaidis, A. Bletsou, C. Zwiener, M. Ibáñez, T. Portolés, R. de Boer, M.J. Reid,
446 M. Onghena, U. Kunkel, W. Schulz, A. Guillon, N. Noyon, G. Leroy, P. Bados, S. Bogialli, D. Stipančev, P.
447 Rostkowski, J. Hollender, Non-target screening with high-resolution mass spectrometry: critical review
448 using a collaborative trial on water analysis, *Analytical and Bioanalytical Chemistry*, 407 (2015) 6237-
449 6255.

450 [30] L. Bijlsma, E. Beltrán, C. Boix, J.V. Sancho, F. Hernández, Improvements in analytical methodology
451 for the determination of frequently consumed illicit drugs in urban wastewater, *Analytical and*
452 *Bioanalytical Chemistry*, 406 (2014) 4261-4272.

453

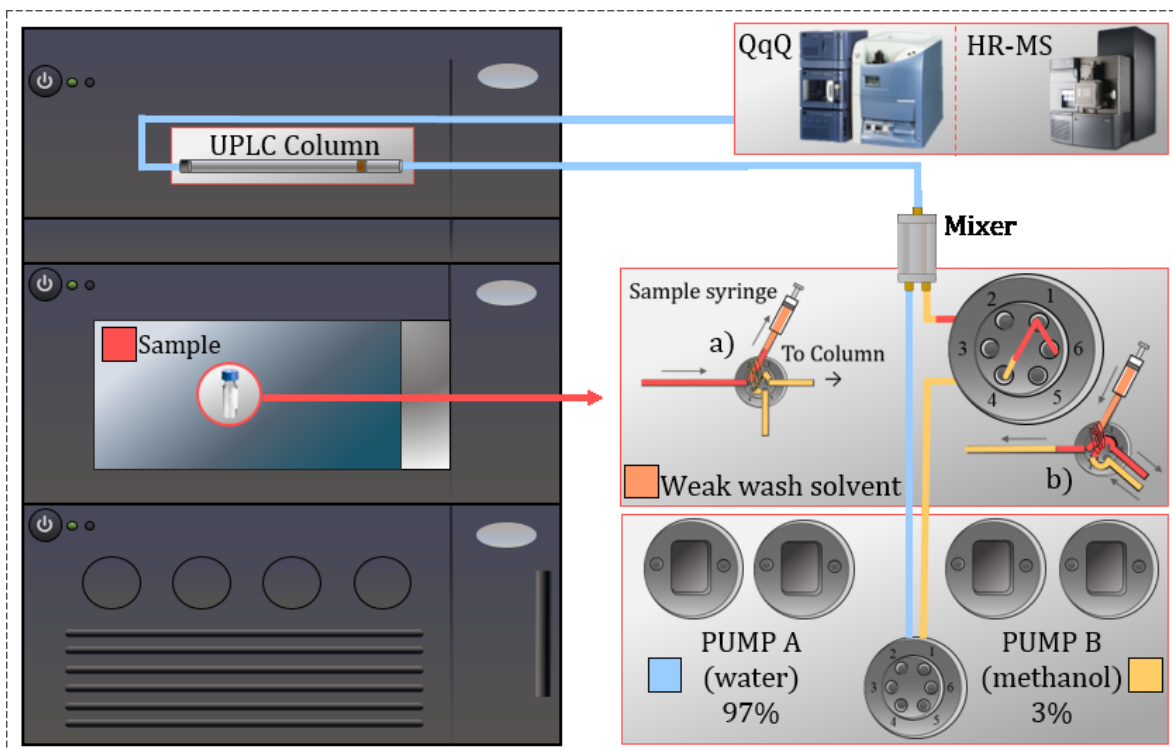
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456 *Figure 1. Illustrative workflow of the analytical procedure using Waters Oasis HLB μElution plate (5 mg,*
 457 *30 μm particle size) for the sample extraction (n=3) and an UPLC-MS/MS for target quantification and*
 458 *UPLC-HRMS for virtual library bank acquisition.*

459



460

461 Figure 2. Schematic representation of post-loop mixing process. Initial mobile phase rate (flow 0.5 mL min^{-1}) is set at 97% A:3% B during the first 5 minutes in order to retain the sample at the head of the
 462 UHPLC column
 463

464

465 Table 1. MS/MS optimized conditions for selected compounds.

Compound	ESI	Retention time	Quantitation			Confirmation
			MRM (Q1 > Q3)	Cone (V)	Collision (V)	MRM (Q1 > Q3)
Amphetamine	+	6.6	136.1 > 91.1	20	15	136.1 > 119.1
Amphetamine-d8	+	6.6	144.1 > 97.1	20	15	-
Methamphetamine	+	7.0	150.1 > 91.1	20	15	150.1 > 119.1
Methamphetamine-d11	+	7.0	161.2 > 127.1	20	15	-
MDMA	+	6.9	194.2 > 163.2	20	15	194.2 > 105.1
MDMA-d5	+	6.9	199.2 > 165.2	20	15	-
Cocaine	+	7.4	304.2 > 182.2	30	20	304.2 > 105
Cocaine-d3	+	7.4	307.2 > 185.2	30	22	-
Benzoyllecgonine	+	5.9	290.2 > 168.2	30	20	290.2 > 105
Benzoyllecgonine-d3	+	5.9	293.2 > 171.2	30	20	-
Cocaethylene	+	8.0	318.2 > 196.2	30	20	318.2 > 82.1
Cocaethylene-d3	+	8.0	321.2 > 199.1	30	20	-
Atenolol	+	6.0	267.2 > 190	25	20	267.2 > 145
Atenolol-d7	+	6.0	274 > 145	30	20	-
Citalopram	+	8.1	325.2 > 262.2	30	22	325.2 > 109.2
Carbamazepine	+	6.5	237.1 > 194.1	25	20	237.1 > 192.1
Fexofenadine	+	5.9	502.3 > 466.3	20	30	502.3 > 171.1
Fexofenadine-d6	+	5.9	508.3 > 472.5	30	30	-
Methylphenidate	+	7.2	243.3 > 84	20	20	243.3 > 174.1
Metoprolol	+	6.7	268.2 > 116	25	20	268.2 > 191
Metoprolol-d7	+	6.7	275.2 > 123.1	28	20	-
Lidocaine	+	7.7	235.3 > 86	25	20	235.3 > 58.1
Lidocaine-d6	+	7.7	241.3 > 86	25	15	-

466

467

468 Table 2. Method performance parameters: linearity, recoveries, repeatability, matrix effect and method
 469 limits of quantification.

	MeOH		Wastewater				LOQ	ILIS used for correction
	Linearity (R^2)	ng mL ⁻¹	Relative recovery (RSD)	Absolute recovery (RSD)	Repeatability (RSD)	Matrix Effects		
			Both in %	Both in %	%	%		
			[100 ng L ⁻¹] n=3	[100 ng L ⁻¹] n=3	[200 ng L ⁻¹] n=6	n=3		
Amphetamine	0.025 - 10 (0.99931)		105 (14)	36 (18)	14.4	80	3.5	Amphetamine-d8
Methamphetamine	0.025 - 10 (0.99941)		94 (10)	95 (3)	9.3	117	1.1	Methamphetamine-d11
MDMA	0.025 - 10 (0.99973)		99 (3)	86 (5)	3.5	104	6.3	MDMA-d5
Cocaine	0.025 - 10 (0.99991)		110 (8)	79 (1)	6.8	117	4.3	Cocaine-d3
Benzoylcegonine	0.025 - 10 (0.99979)		103 (5)	86 (14)	4.3	87	2.9	Benzoylcegonine-d3
Cocaethylene	0.025 - 10 (0.99997)		98 (3)	86 (1)	3.4	118	1.0	Cocaethylene-d3
Atenolol	0.025 - 10 (0.99871)		104 (12)	87 (3)	11.1	55	4.4	Atenolol-d7
Citalopram	0.025 - 10 (0.99984)		92 (10)	87 (7)	11.3	96	1.1	Cocaethylene-d3
Carbamazepine	0.025 - 10 (0.99937)		104 (9)	93 (15)	11.3	102	0.2	Metoprolol-d7
Fexofenadine	0.025 - 10 (0.99980)		96 (8)	90 (12)	8.8	21	5.6	Fexofenadine-d6
Methylphenidate	0.025 - 10 (0.99979)		105 (7)	91 (11)	4.9	78	1.9	Cocaine-d3
Metoprolol	0.025 - 10 (0.99954)		109 (16)	94 (3)	12.6	104	2.1	Metoprolol-d7
Lidocaine	0.025 - 10 (0.99989)		100 (3)	92 (5)	3.6	113	0.3	Lidocaine-d6

470

471

472 Table 3. Concentrations of the target compounds quantified in 10 wastewater samples from Oslo in
 473 2016 (ng L⁻¹).

474

		Wastewater Concentration (ng/L)									
Date		17-19/06/16	20/06/16	21/06/16	22/06/16	23/06/16	24-26/06/16	27/06/16	28/06/16	29/06/16	30/06/16
Compound		Weekend	Monday	Tuesday	Wednesday	Thursday	Weekend	Monday	Tuesday	Wednesday	Thursday
Amphetamine		459	282	227	372	426	594	393	372	402	349
Methamphetamine		447	300	250	375	398	480	386	353	395	354
MDMA		117	61	28	45	44	145	78	44	38	35
Benzoyllecgonine		644	340	236	405	535	718	495	420	456	371
Cocaine		300	119	108	195	257	306	187	194	195	176
Cocaethylene		16	6	4	7	10	16	9	8	9	8
Atenolol		42	30	25	39	44	38	33	43	48	32
Citalopram		65	58	35	60	66	59	58	66	71	55
Carbamazepine		1379	1241	888	1315	1433	1277	1168	1200	1389	1091
Fexofenadine		205	165	117	167	178	182	142	166	165	165
Methylphenidate		185	167	140	205	263	204	215	232	232	177
Metoprolol		6	5	3	7	10	9	7	7	7	5
Lidocaine		89	87	55	71	85	83	72	77	108	78

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