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1 **Application of wastewater-based epidemiology to investigate stimulant drug,**
2 **alcohol and tobacco use in Lithuanian communities**

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5
6 **Abstract**

7 WBE was applied to evaluate illicit drug (i.e. amphetamine, cocaine, MDMA and methamphetamine),
8 alcohol and tobacco use in three Lithuanian cities in 2018 and 2019. Considerable concentrations of
9 methamphetamine and MDMA were found in the three locations, suggesting a specific Lithuanian
10 consumption pattern. Yet, unexpected high concentrations of amphetamine (> 4 µg/L) were detected
11 in two samples of Kaunas in 2018. Through the use of chiral analysis and non-target and suspect drug
12 precursor compound screening, these extreme values were confirmed to be the result of direct
13 disposal of amphetamine in the sewers. Furthermore, substantial alcohol use was measured in the
14 three investigated catchment populations of Lithuania with almost 4 standard drinks/day/inhabitant
15 aged 15+ on average in 2019. For tobacco, an average of 5.6 cigarettes/day/inhabitant aged 15+ in
16 2019 was reported with large discrepancies between WBE figures and sales data, potentially
17 highlighting illegal trade of tobacco products.

18

19 **Highlights**

- 20 - Illicit drug, alcohol and tobacco use in Lithuania was assessed through wastewater-based
21 epidemiology
- 22 - Methamphetamine and MDMA were the most abundant substances used
- 23 - State-of-the-art analytical techniques revealed a dumping event of amphetamine
- 24 - Measured alcohol use was almost twice the European average
- 25 - Some discrepancies with other data sets on nicotine use were observed, potentially suggesting illegal
26 trade

27 **1. Introduction**

28 The population health status in Lithuania has improved for the past decade, but still remains much
29 lower compared to most European countries¹⁻³. Even though the life expectancy in Lithuania is on the
30 rise, it still remains the lowest in the European Union (EU)^{2,3}. The health status and the related gender
31 gap (with a life expectancy 10 years higher for women) are closely linked to behavioural risk factors
32 (e.g. alcohol consumption, tobacco smoking, illicit drug use, low physical activity, diet) which attribute
33 to almost 40% of the overall burden of disease in Lithuania³.

34 Data from the World Health Organization (WHO) suggests that the per capita alcohol consumption
35 (APC) in the Baltic States is 1.5 times higher than the EU average, with Lithuania situated among the
36 heaviest-drinking countries worldwide^{3,4}. As a result, many of the leading causes of death in Lithuania
37 (e.g. cardiovascular diseases, liver diseases, accidental poisoning and road traffic accidents) are
38 associated with high alcohol use and the alcohol-related mortality rate is estimated to be much higher
39 compared to the EU average³⁻⁵. Smoking is also an important health issue in Lithuania; in fact, 20% of
40 the Lithuanian population smoked daily in 2014² despite the strengthened tobacco regulations during
41 the past decade³. Even though the number of adults smoking tobacco in Lithuania has dropped sharply
42 to below EU average, 33.9% of men still remain daily smokers³.

43 . General population survey (GPS) data shows that approximately 11.5% of Lithuanian adults used an
44 illicit drug in their lifetime, with drug use being highest among young adults aged 15-34 years⁶.
45 Additionally, methylenedioxymethamphetamine (MDMA) was used most frequently as a stimulant in
46 2016 based on survey data, followed by other amphetamines (i.e. amphetamine and
47 methamphetamine) and cocaine⁶. Furthermore, Lithuania's death rates from drug overdoses is twice
48 the EU average^{7,8}. While the production of stimulants is considered to be limited, methamphetamine
49 proves to be the most common illicit drug produced in Lithuania, as reflected by its domestic use and
50 police reports on methamphetamine laboratories detections in the past. Lithuania is considered to be

51 a transit country for illicit drug trafficking between Eastern and Western European and Scandinavian
52 countries⁹.

53 Even though a lot of valuable epidemiological information in Lithuania is already available at this
54 moment, there are still some knowledge gaps, especially regarding illegal substance use. Current
55 figures on the consumption of legal and illegal substances are mainly obtained through sales data and
56 GPS, which are inherently linked to some limitations and challenges. A main limitation of the GPS is
57 the infrequency of data reporting (i.e. not on a yearly basis) and reporting and concealment bias
58 regarding substance use experiences. A limitation associated with sales data is that the locality
59 between where these substances are sold and the consumption occurs might not be the same and
60 countries with low taxation rates are often associated with high sales figures. As such, alcohol and
61 cigarettes could be imported from neighbouring countries in order to avoid growing taxation rates in
62 Lithuania. Additionally, data on substances produced, distributed and sold outside the formal channels
63 under governmental control (i.e. home-made and illegal production, illegal trafficking, internet
64 sales,...) are not included in official sales statistics. According to the WHO up to 8% of total per capita
65 alcohol consumption in Lithuania is not recorded in official sales statistics⁴. Additionally, smuggling of
66 tobacco products from Belarus and supply of tobacco heating products from Ukraine is a well-known
67 public health concern in Lithuania¹⁰⁻¹², however the extent needs further exploration. In this light, new
68 complementary data sources are needed to provide actual information on intra-country differences
69 and trends in the amounts of substances consumed.

70 Over the past decade, wastewater-based epidemiology (WBE) has become a reliable and
71 complementary approach to monitor and back-estimate illicit drug use in populations¹³⁻¹⁷. Within this
72 methodology, human metabolic excretion products are collected, pooled and transported in the
73 wastewater system, providing valuable information on the amount and type of substances used by
74 defined population groups¹⁶. Concentrations of biomarkers in wastewater are multiplied with daily
75 wastewater flow rates and divided by the population served by the wastewater treatment plant

76 (WWTP) to obtain population-normalized mass loads (mg/day/1000 inhabitants). This allows
77 comparison of results across different locations and at different time points. Proof-of-concept studies
78 have been performed to show that WBE is further suitable to assess community health. For example,
79 WBE was applied to monitor alcohol, tobacco and pharmaceutical use¹⁸⁻²⁶, to measure endogenous
80 substances of disease or health and to estimate the exposure to emerging contaminants, such as
81 pesticides or flame retardants^{27,28}. WBE can thus provide complementary epidemiological information
82 and fill in some of the current knowledge gaps regarding the public health status in Lithuania. WBE
83 can be used to continuously and in near real-time monitor the evolution of lifestyle-related indicators
84 in different communities²⁹. This unique feature makes WBE an excellent early-warning information
85 system able to capture quickly developing changes in substance use. This characteristic provides
86 policy-makers crucial information to allow timely decision-making and evaluating the effectiveness of
87 new guidelines and national and local initiatives. By monitoring at a high spatial resolution, WBE offers
88 a possibility to assess intra-country differences in the consumption of illicit drugs alcohol and
89 tobacco^{15,18,20}. Furthermore, WBE could be particularly useful to deliver information on illegal
90 consumption and trade of substances. However, WBE is not able to provide any information on
91 individual consumption patterns and socio-demographics of the user.

92 The manuscript reports on the investigation of substance use (i.e. stimulants, alcohol and tobacco) in
93 Lithuania with a focus on intra-country differences and temporal variations by a WBE approach. The
94 obtained data can be used to gather more evidence on the lifestyle-related health status of the
95 Lithuanian population but also to put this in a broader European perspective.

96 **2. Materials and Methods**

97 2.1. Reagents and Materials

98 The analytes of interest (purity $\geq 99\%$) and their deuterated analogues used as internal standards (IS)
99 were obtained from Cerilliant (Round Rock, Texas, USA), Toronto Research Chemicals (Toronto, ON,
100 Canada) and Athena Enzyme Systems (Baltimore MD, USA). Reference standards and deuterated

101 reference standards were of analytical grade and purchased as neat powder or as solutions of 1 mg/mL
102 or 100 g/mL in methanol (MeOH) or acetonitrile (AcN). Dilutions and working mixtures with
103 concentrations ranging between 0.05 and 100 g/mL were prepared in MeOH. Analytical grade
104 hydrochloric acid (HCl), ammonium hydroxide (NH₄OH), formic acid (HCOOH), acetic acid (CH₃COOH)
105 and ammonium acetate and LC grade AcN and MeOH were purchased from Merck (Darmstadt,
106 Germany). Milli-Q ultrapure water was obtained by purifying demineralised water in an Elga LabWater
107 Purelab Flex system (Veolia Water Solutions & Technologies Belgium, Tienen, BE). Oasis MCX (60 mg,
108 3 mL) solid-phase extraction (SPE) cartridges were purchased from Waters (New Bedford,
109 Massachusetts, USA). A Supelco Visiprep SPE Vacuum Manifold 24-port model with a self-cleaning dry
110 vacuum system Welch 2023 was used for the loading of the sample on the cartridge and the drying of
111 the cartridges. Safe-lock tubes (1.5 mL and 2 mL) were obtained from Eppendorf (Rotselaar, Belgium)
112 and centrifugal filters (0.20 µm and 0.45 µm) containing modified nylon were acquired from VWR
113 (Leuven, Belgium).

114 2.2. Sampling and analysis

115 2.2.1. Sampling

116 Influent wastewater (IWW) samples were collected from three Lithuanian cities (Figure 1) (i.e. Kaunas,
117 Klaipeda and Vilnius), covering approximately 35% of the Lithuanian population³⁰. 24-h composite
118 IWW samples were collected time-proportionally in 2018 and 2019 for at least seven consecutive days
119 (see Table 1). Aliquots of 500 mL were immediately frozen after collection and stored at -20 °C until
120 analysis to prevent degradation of biomarkers³¹. No special events were reported within the sampling
121 period in the different locations. pH of the IWW samples ranged between 7.4 and 8.5 and temperature
122 between 13 and 15°C. Average residence time was in all locations less than 24 hours.

123 2.2.2. Sample preparation and instrumental analysis

124 Sample preparation was done accordingly to previously validated bioanalytical methods. Performance
125 criteria of these bioanalytical methods (i.e. accuracy, precision,...) met the criteria for method

126 validation provided by the European Medicines Agency (EMA) guidelines. Either direct injection or
127 solid-phase extraction (SPE) were employed based on the biomarker concentrations in IWW. A detailed
128 description of the well-established bioanalytical methods is given in the Supplementary Information
129 (S1, Table S1 and S2). Extraction of benzoylecgonine (BE), amphetamine (AMP), methamphetamine
130 (METH) and 3,4-methylenedioxymethamphetamine (MDMA) in IWW was done according to van Nuijs
131 et al.³² Sample preparation for the analysis of ethyl sulphate (EtS) and cotinine (COT)/hydroxycotinine
132 (COT-OH) in IWW was performed according to a validated method by Boogaerts et al and Lai et al,
133 respectively^{18,21} Multi-level calibration curves with final concentrations ranging between 1-3000 ng/L
134 were constructed for each analyte with different working standard solutions and a fixed amount of
135 deuterated analogue in methanol. For analyte confirmation, the quantifier/qualifier ratio must not
136 differ more than $\pm 15\%$ and the relative retention time must not differ more than 2.5%. Quality control
137 was performed by in-house QA/QC measures and for illicit drugs through participation in an inter-
138 laboratory exercise provided by Sewage Analysis CORe group Europe (SCORE)³³.

139 .

140 2.2.3. Confirmation of a direct dumping event by enantiomeric analysis and non-target and suspect
141 screening

142

143 Enantiomeric analysis and non-target and suspect screening were only considered when a potential
144 dumping event was suspected based on daily mass load fluctuations. This was only the case for the
145 sampling week in 2018 in Kaunas, where two extremely high values for AMP (1000-fold higher than
146 baseline loads) were detected. For the other locations and years we observed daily variations in
147 population-normalised mass loads that could be attributed to human consumption; no indications for
148 dumpings could be found. Other biomarkers were not considered for enantiomeric analysis and non-
149 target and suspect screening for the same reason. Additionally, this should only be considered when
150 the parent compound is used as human biomarker.

151 The chiral signatures of AMP in influent wastewater were verified with chiral liquid chromatography
152 coupled to tandem mass spectrometry to distinguish between consumption and direct disposal of
153 AMP. Chiral analysis was undertaken according to the methodology described elsewhere (also in the
154 Supplementary information)^{34,35}.

155

156 The enantiomeric fraction (EF) was calculated using the following equation (1):

$$157 \quad EF = \frac{S(+)}{(S(+)+R(-))} \quad (1)$$

158 where

$$159 \quad S(+) = \frac{\text{peak area of analyte } S(+)\text{ - enantiomer}}{\text{peak areas of internal standard } S(+)\text{ - enantiomer}}$$

$$160 \quad R(-) = \frac{\text{peak area of analyte } R(-)\text{ - enantiomer}}{\text{peak areas of internal standard } R(-)\text{ - enantiomer}}$$

161

162 EF equals 0.5 in the case of a racemate, whilst 1 or 0 in the case of the enantiopure compound.

163

164 Non-target and suspect screening of drug precursors was previously described in Emke et al³⁶. An in-
165 house suspect list containing relevant compounds (i.e., (pre-)precursors, intermediates, impurities, by-
166 products) was compiled from available literature for both MDMA and AMP. The latter contained 197
167 and 70 compounds for MDMA and AMP, respectively. Day 7 and 8 of Kaunas from 2018 were
168 considered to be from non-consumption origin. Hence the ratio between the consumption and non-
169 consumption group was determined. A minimum peak intensity of 500'000 counts was used for peak
170 picking, together with a log-fold 2 change greater than 4 and a group ratio greater than 20. A mass
171 tolerance of 3 ppm and retention time shift of 1 min were used for feature detection. Library searches
172 were conducted against *mzCloud* (HighChem Ltd, Slovakia), *mzVault* (Thermo Fisher Scientific Inc, USA)
173 (with the *mzVault May 2018* library), and *Chemspider* (Royal Society of Chemistry, USA) (with *EAWAG*
174 *biocatalysis/biodegradation*, *EPA DSSTox*, *EPA toxcast*, *Drugbank*, *ACToR*, and *FDA UNII – NLM*
175 databases), whilst suspect screening was performed using the abovementioned in-house list.

176 2.2.4. Back-calculations and data analysis

177

178 Measured concentrations of all biomarkers (expressed in ng/L) were multiplied by daily wastewater
179 flow rates (L/day) recorded in the WWTPs and divided by the population sizes to obtain population-
180 normalized mass loads (mg/day/1000 inhabitants). These can be considered as a proxy for
181 consumption of the parent compound. The normalization for population enables the comparison of
182 consumption patterns across different locations and different time points. For the illicit drugs, back-
183 calculations to population-normalized mass loads were performed with no further back-calculations
184 to doses. For alcohol and tobacco, mass loads were further transformed into per capita daily standard
185 doses to allow a brief comparison between WBE data and official sales figures.

186

187 In order to transform standard doses of alcohol and tobacco, excretion rates and molar mass ratios
188 were taken into account^{18,21,24,25,37}. A conversion factor of 3049 was applied to transform population-
189 normalized mass loads of EtS in per capita loads of alcohol. In order to obtain standard alcohol doses,
190 daily per capita loads of alcohol were divided by the alcohol content in a standard alcohol drink (9.86
191 g), as illustrated by Equation 1.

192

193
$$\text{Daily per capita standard alcohol doses} = \frac{\text{g EtS per day per capita} \times 3049}{9.86 \text{ g}}$$

194 *Equation 1 Back-calculation of per capita standard alcohol doses*

195 In this study, population-normalized mass loads of nicotine were derived from COT and COT-OH. A
196 correction factor of 3.13 and 2.31 were used respectively to derive population-normalized mass
197 loads of nicotine, as illustrated by Equation 2. Population-normalized, COT and COT-OH derived
198 estimates of nicotine were averaged and divided by the average amount of nicotine per cigarette to
199 obtain daily cigarettes consumption per capita.

200
$$\text{mg nicotine per day per capita} = \frac{(\text{mg cotinine per day per capita} * 3.13) + (\text{mg COT} - \text{OH per day per capita} * 2.31)}{2}$$

201
$$\text{Daily per capita standard cigarette doses} = \frac{\text{mg nicotine per day per capita}}{0.9 \text{ mg}}$$

202 *Equation 2 Back-calculations of per capita standard cigarette doses*

203 To compare WBE figures with the other Lithuanian data sources only inhabitants aged 15+ were
204 included (i.e. 84.9% of Lithuanian population), which resulted in standard doses per capita aged 15+³⁰.
205 Results from the different locations were combined to estimate an annual national amount of
206 consumed substances. Weighted averages were used for this calculation in order to take the
207 proportions of the populations covered by the different catchments into account. Furthermore, the
208 WBE estimates reported in this study were compared with WBE results from other European countries
209 collected in a similar manner^{18,21,24,37-43}. In order to compare results with these figures, the population
210 was not normalized for age 15+.

211
212 Statistical analysis was performed with Prism version 8.3.1. (GraphPad Software, California, USA). A
213 Shapiro-Wilk normality test was applied to test if data was distributed normally in order to test if
214 parametric tests were appropriate or not. Variations between years and locations were assessed by
215 applying a parametric One-way Anova test, followed by a Dunn's post hoc test or the non-parametric
216 equivalent depending on the normal distribution ($\alpha=0.05$).

217

218 **3. Results and discussion**

219 3.1. Illicit drug consumption

220 3.1.1. Intra-country differences in illicit consumption

221 The population-normalised mass loads of illicit drugs in the three Lithuanian catchments are shown in
222 Figure 2.

223 Daily variations in population-normalised mass loads of all biomarkers are given in Table S4 and Figure
224 S1. For COC and MDMA, no significant spatial differences were found in the consumption patterns in
225 both years, as illustrated by Figures 2B and C. In 2018 no significant spatial differences were found in

226 the consumption of AMP in the investigated catchment areas (Figure 2A). However, the use of AMP
227 was in 2019 significantly higher in Kaunas compared to Klaipeda. In addition, METH consumption was
228 higher in Kaunas and Vilnius compared to Klaipeda in both years (Figure 2D), which was in line with
229 GPS data⁶. It should be noted that AMP found in the sewer could also originate from METH
230 consumption since AMP is a human metabolic excretion product. However, this fraction is limited since
231 METH is only excreted in urine as AMP to a minor extent.

232 We hypothesize that the higher use of AMP and METH in Kaunas and Vilnius is due to the closer
233 proximity of these cities to production sites and drug trafficking routes compared to Klaipeda.
234 Additionally, it should also be noted that tourism is considerably higher in Kaunas and Vilnius
235 compared to Klaipeda, which could potentially contribute to differences in the consumption patterns
236 of METH.

237

238 3.1.2. Inter-country differences in illicit drug consumption

239 Population-normalized mass loads for the stimulants were compared with the results from other
240 European WBE studies. MDMA consumption in Lithuania proved to be similar to Central Europe and
241 Scandinavian countries, but was lower compared to Western European countries, as illustrated by
242 Figure 3. MDMA is mainly consumed by young adults which could explain the present population-
243 normalised loads of MDMA in the investigated urbanized areas^{8,44}. Lower consumption rates in
244 Lithuania in contrast to Western Europe could be the result of the higher availability of MDMA in
245 Western Europe (i.e. high abundance of illegal production sites of MDMA in Belgium and the
246 Netherlands), as reported in the EU Drug Markets Report from the EMCDDA and Europol⁴⁵.

247

248 WBE shows that COC, in contrast to Western-European countries, proved to be a less frequently used
249 illicit drug in Lithuania (Figure 3)^{38,44}. Population-normalised mass loads of BE were considerably higher
250 compared to Finland. The presence of COC could be due to the the economic growth in Lithuania which
251 resulted in cocaine entering the illicit drug market. Western-European countries, however, remain the

252 primary import countries for wholesale cocaine trafficking with the Ports of Antwerp and Rotterdam
253 as the most important transit ports which is reflected by the higher availability in Western Europe
254 compared to Central and Eastern Europe⁴⁶.

255
256 AMP consumption was lower compared to Western-European countries and Scandinavian countries
257 and similar to Central Europe (Figure 3)^{38,44}. Although population-normalized mass loads of AMP were
258 rather low in a European perspective, unexpected high mass loads of AMP were found in two
259 wastewater samples from Kaunas from 2018 (Figure 4). The observed load on day 7 was 1000-fold
260 higher compared to the rest of the week. These extreme values were suspected to be the result of
261 direct disposal of AMP in the wastewater system. While illicit drug production in Lithuania is
262 considered limited, a few amphetamines production sites have been dismantled in recent years which
263 could explain its domestic use⁸. In addition, consumption of amphetamines is high due to Lithuania's
264 position as part of a trafficking route. It has been reported by the EMCDDA that significant production
265 and trafficking of amphetamines takes places in North-East European countries including Lithuania,
266 Estonia, Latvia and Poland⁴⁷. Amphetamines available in Norway, Finland and Sweden are almost
267 exclusively produced from benzylmethylketone (BMK) by means of a reductive amination, the so called
268 Leuckart route and trafficked as racemate from the Baltic States. Non-target and suspect screening of
269 these two wastewater samples indicated the presence of Leuckart-specific intermediates (e.g. N-
270 formylamphetamine) and impurities (e.g. 4-benzylpyrimidine, N,N-di-(b-phenylisopropyl)formamide,
271 N,N-di-(b-phenylisopropyl)amine), which are formed when BMK is being used as a precursor for AMP
272 synthesis through the Leuckart reductive amination route³⁶. A list of all possible structures correlating
273 with the high peak load of amphetamine found in these samples related to AMP production was given
274 in Table 2. Chiral profiling of AMP revealed that AMP found in the IWW samples from Kaunas was
275 racemic on the days following the suspected dumping event (Figure 4). Castrignano et al. revealed that
276 consumption of racemic AMP favours the excretion of the R-(-)-enantiomer (EF<0.5)³⁵. This further

277 corroborates direct disposal of racemic (unconsumed) AMP in the wastewater system, possibly by
278 criminal organizations prior to a police raid.

279

280 It is also important to note that these aberrant loads of AMP were excluded from the spatio-temporal
281 comparison. However, depending on the distance between the dumping event and the WWTP, it might
282 take several days for the dumped AMP to reach the inlet of the WWTP. For this reason, we should be
283 careful with interpreting the population-normalised mass loads observed within this sampling period.
284 A better approach would be to analyze a different 'normal' week (i.e. without special events) for the
285 spatio-temporal analysis, however, no IWW samples are available anymore for this retrospective
286 analysis.

287

288 Although the METH market in Europe is relatively low on a global scale, substantial loads of this
289 stimulant were measured in all locations sampled in Lithuania. This is different to the consumption
290 patterns observed in most Western-European countries and similar to Central Europe albeit in a lesser
291 proportion. This might also be attributed to the considerable domestic production of METH and
292 trafficking in the North-Eastern parts of Europe⁴⁸.

293

294 3.1.3. Temporal changes in illicit drug consumption

295 The use of COC was stable between 2018 and 2019. AMP consumption increased significantly in 2019
296 in Kaunas and Vilnius. The use of MDMA also statistically increased in 2019 in Kaunas and Klaipeda,
297 while the use of METH in Kaunas statistically decreased in 2019. Additionally, weekly variations (Figure
298 S1) were also observed in the consumption of the different stimulants with the highest population-
299 normalised mass loads retrieved during the weekends.

300

301 In Lithuania seized amounts of cocaine, amphetamine and MDMA increased respectively from 13.8 kg
302 to 16.1 kg, 6.5 kg to 23.6 kg and 17.4 to 282 kg between 2018 and 2019, while the seized amounts of

303 METH decreased drastically from 36.9 kg to 4.4 kg. Extrapolated national population-normalised loads
304 of BE, AMP and MDMA also increased slightly between 2018 and 2019, however, not to the same
305 extent compared to seizure data. In contrast to seized amounts of METH, extrapolated national
306 population-normalised mass loads of METH remained stable between 2018 and 2019⁴⁹. It has to be
307 noted that seizure data and WBE data do not reflect the same aspect of the illicit drug status (i.e. law
308 enforcement vs actual consumption) and it highlights the need of combining complementary data
309 sources to obtain a more complete picture on the illicit drug situation.

310

311 3.2. Alcohol consumption

312 3.2.1. Intra- and inter country differences in alcohol consumption

313 Daily alcohol consumption ranged between 1.7 and 7.4 standard drinks/day/capita aged 15+ in 2018
314 and 2019 among the different catchments. Spatial differences were observed in 2018 with Vilnius
315 having a consumption per capita 30% higher than Kaunas. In 2019, alcohol consumption in Vilnius and
316 Klaipeda was higher compared to Kaunas. Alcohol use in Vilnius and Klaipeda did not differ
317 significantly. In Vilnius the age group of inhabitants between 25 and 44 years old is also considerably
318 larger (36% of the population of Vilnius) compared to Kaunas (29% of the population of Kaunas) and
319 Klaipeda (26% of the population of Klaipeda). According to the GPS, the highest prevalence of alcohol
320 use is among the 25-44 years old age group. Another possible explanation could be related to the
321 availability of alcohol in Klaipeda and Vilnius compared to Kaunas. The density of licences issued for
322 alcohol sales is higher in Klaipeda and Vilnius compared to Kaunas.

323

324 Alcohol consumption among the different Lithuanian cities reported in this study still remains
325 significantly higher in comparison with the European average reported by the WHO. This is further
326 evidenced by other European WBE studies in which alcohol consumption was monitored through the
327 measurement of EtS in wastewater^{18,24,39,40,43}. It should be noted that all these WBE studies reported
328 amounts consumed between 2012 and 2015 and temporal changes in alcohol use may therefore

329 complicate the comparison with these measurements. While spatial differences between our study
330 and the other WBE studies are obvious, the overall order of magnitude is in good agreement.

331 3.2.2. Temporal changes in alcohol consumption

332 No significant temporal differences in consumption patterns between 2018 and 2019 were observed
333 for Kaunas and Vilnius. In Klaipeda, alcohol consumption statistically increased with 53% in 2019.
334 Alcohol use in Lithuania still remains problematic despite the new alcohol regulations (i.e. restricting
335 sales times, banning alcohol advertising and increasing the legal age for alcohol consumption to 20
336 year) that went into effect in January 2018³. These regulations were deemed necessary in response to
337 the persistently high consumption rates after introducing policy changes in 2007-2008, which
338 restricted advertising and sales and increased taxes.

339 3.2.3. Comparison between WBE estimates and other data sources

340 Consumption estimates for alcohol based on WBE were compared with consumption rates acquired
341 from the WHO which includes recorded and unrecorded alcohol per capita consumption⁴. Unrecorded
342 alcohol consumption is regarded as alcohol consumption not accounted for in official taxation and
343 sales statistics usually produced, distributed and sold outside formal governmental channels. However,
344 the monitoring of unrecorded consumption continues to be challenging for national surveillance
345 systems and is mostly based on self-reported survey data, which is subject to concealment and
346 reporting bias. This could lead to underestimation of the illegal alcohol consumption per capita.
347 Nation-wide estimates based on WBE data in 2018 and 2019 were in line with data from the WHO, as
348 illustrated by Figure 5A. No significant differences were found between the WHO estimate and the
349 weighted mean of each year. It should be noted that the latest numbers from the WHO were from
350 2016 while wastewater samples originated from 2018 and 2019 and that consumption rates obtained
351 from WHO data might be different in 2016 compared to 2018 and 2019. However, more recent data
352 was not available at the time of the investigation.

353 3.3. Tobacco consumption

354 3.3.1. Intra-country and intercountry differences in tobacco consumption

355 Daily cigarette consumption ranged between 3.5 and 10.6 cigarettes/day/capita aged 15+. In 2018, no
356 significant differences were observed in the cigarette consumption between all measured locations. In
357 2019, however, tobacco use was significantly higher in Vilnius and Klaipeda compared to Kaunas.
358 Currently, a specific explanation for this is yet to be found.

359 Population-normalized mass loads of nicotine were derived from COT/COT-OH and were used to
360 compare smoking between European countries. Population-normalized mass loads of nicotine found
361 in this study were in line with other European WBE studies, which further supports the applicability of
362 the proposed methodology (Figure 6B). It should be noted that only a limited number of WBE studies
363 focussed on tobacco consumption in Europe and that most of the WBE studies focussing on tobacco
364 are from Australia and China.

365 3.3.2. Temporal changes in tobacco consumption

366 Over the past few years, the tobacco regulation in Lithuania has become stricter with the introduction
367 of indoor smoking bans and the prohibition of smoking in cars with children or pregnant women. In
368 spite of these tighter regulations and governmental efforts to diminish smoking, a significant decrease
369 in tobacco consumption in 2019 was only observed in Kaunas. Tobacco consumption in Vilnius and
370 Klaipeda remained stable throughout the entire sampling period.

371 3.3.3. Comparison between WBE estimates and other data sources

372 Measured cigarette consumption based on WBE loads was significantly higher compared to cigarette
373 consumption based on sales data (also includes self-made cigarettes)⁵⁰. It should be noted that
374 national estimations are only based on measurements in three locations and that different
375 consumption rates might be observed in other areas. Another limitation is that the latest available
376 estimates for Lithuania from the Tobacco Atlas were from 2016 and this might not be representative

377 for the sampling period due to temporal variations. Another reason for the discrepancies between the
378 national estimates based on WBE and the sales statistics could be related to the conversion of
379 population-normalised mass loads of COT and COT-OH to the number of cigarettes, especially with
380 regards to the back-calculation factors and the varying nicotine content in cigarettes.

381 Nicotine biomarkers found in wastewater could also be derived from human exposure to nicotine from
382 other sources, such as tobacco heating products (e-cigarettes, water pipes,...), nicotine patches and
383 gums,..., which could further lead to discrepancies between sales statistics and WBE estimates .
384 Additionally, purchase and consumption locations might not be the same. Cigarettes may also be
385 (illegally) imported from neighbouring countries (e.g. Belarus) to avoid increasing taxation rates in
386 Lithuania¹¹. Data on illegal trade of tobacco products is not covered by these sales figures which could
387 lead to an underestimation of the actual consumption.

388 Finally, cigarette buds might end up in the sewer system and could contain unconsumed amounts of
389 nicotine (especially runoff in combined sewers). It is also possible that these remains could be
390 transformed into COT and COT-OH in presence of microbiota present in the sewer system^{51,52}.

391 **4. Study limitations**

392 In general, WBE has some inherent limitations and uncertainties with regards to biomarker stability,
393 real-time population, sampling frequency, chemical analysis and pharmacokinetic information on the
394 different substances (mainly metabolism and excretion)⁵³. In this particular study, time-proportional
395 sampling was used for the collection of daily 24-h IWW samples. In contrast to flow-proportional
396 sample collection, time-proportional sampling mode is not weighted properly for sewer flows.
397 However, a high frequency (<20 minutes) was used to compile the daily IWW samples. Therefore, it is
398 reasonable to assume that the applied sampling mode and frequency are suitable to accurately capture
399 average biomarker concentrations over the 24-h period. Human biomarkers could potentially be
400 transformed by biological and chemical processes that take place during the in-sewer transport from
401 the place of excretion to the WWTP⁵⁴. Multiple studies have proven the in-sewer stability of the

402 analytes of interest in wastewater (for at least 24 hours at pH 7.5 and 20°C)^{31,51,55} However, EtS is found
403 to degrade considerably in real rising main sewer and simulated rising main and gravity sewer
404 conditions^{51,55}. The average residence time reported in this study was less than 24 hours for all
405 locations of interest. Additionally, in-sewer pH and temperature measured in the locations of interest
406 were comparable to the studies above. For this reason, in-sewer (bio)transformation of illicit drug and
407 tobacco biomarkers can be considered negligible. However, the uncertainty of potential in-sewer
408 degradation of EtS could contribute to discrepancies between WBE and other epidemiologic data.

409 In this WBE study, fixed population equivalents were used in order to obtain population-normalised
410 mass loads while in- and efflux of people within the catchments (e.g. tourism, commuting ...) might be
411 substantial. In the future, we should therefore assess the possibility of using dynamic population size
412 proxies (e.g. online measurements of ammonium in wastewater, mobile phone data,...) to cope with
413 fluctuating population dynamics within the different catchments^{52,56,57}.

414 Additionally, this study only investigated a snapshot of substance consumption during one 'normal'
415 week of sampling per location and per year and subsequently extrapolated to national amounts.
416 Consumption rates might be different in the sampling period compared to the rest of the year due to
417 seasonal and weekly variations, which increases the overall uncertainty with regards to the between-
418 year trends. However, the samples were collected in a 'normal' week with no particular events
419 occurring which might bias the findings within this week. All monitored catchments also have more
420 than 100,000 inhabitants, which might not be representative for less populated (rural) areas. While we
421 cover 35.6% of the Lithuanian population, it cannot be ignored that socio-demographics of these
422 municipal areas might also differ significantly from other parts of Lithuania.

423

424 In order to calculate standard doses of alcohol and daily cigarette consumption, excretion factors were
425 derived from pharmacokinetic studies with a limited number of participants which might not reflect
426 the average excretion profile in large populations. In order to acquire more accurate absolute

427 estimates, refinement of these excretion factors should be considered⁵⁸. However, the primary goal of
428 WBE is to investigate spatio-temporal trends in the consumption patterns of different substances and
429 for this purpose population-normalised mass loads prove to be appropriate.

430 **5. Conclusions**

431 The results of this study illustrate the complementarity of WBE to evaluate substance use at the
432 population level. While WBE has been widely adopted in literature and well established for the
433 measurement of illicit drugs in Europe, its application for the measurement of alcohol and tobacco in
434 an European perspective has been more limited. At this moment, WBE is an underemployed tool for
435 monitoring substance use in Eastern European countries and still has a lot of potential in this
436 geographical area to provide a complementary measurement of substance use.

437 METH and MDMA where the most abundant illicit drugs used. This study revealed a dumping event of
438 AMP in the sewer system, which was verified by chiral analysis of AMP and target and non-target
439 screening for drug precursors. Alcohol use in Lithuania proved to be higher compared to other
440 European countries and it has slightly increased from 2018 to 2019. In contrast, the use of tobacco
441 remained quite stable throughout both years and was similar to the amounts reported in other
442 European WBE studies. Interestingly, this study found significant discrepancies between WBE data and
443 sales statistics of tobacco. A potential hypothesis could be the illegal trade of cigarettes and tobacco
444 heating products from neighboring countries (e.g. Belarus, Ukraine). However, more research is
445 needed with regards to conversion factors to decrease the uncertainty associated with WBE back-
446 calculations.

447 **Conflicts of interest**

448 There are no conflicts to declare.

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454 861602).

455 **Graphical abstract**



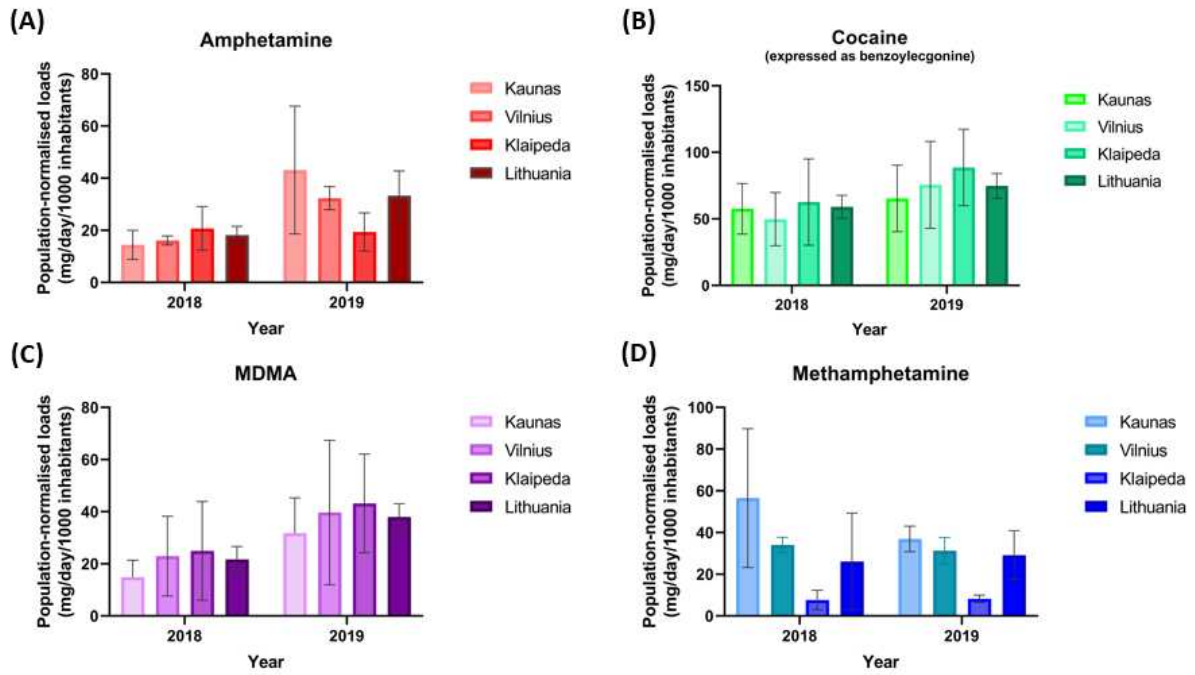
456

457 **Figures**



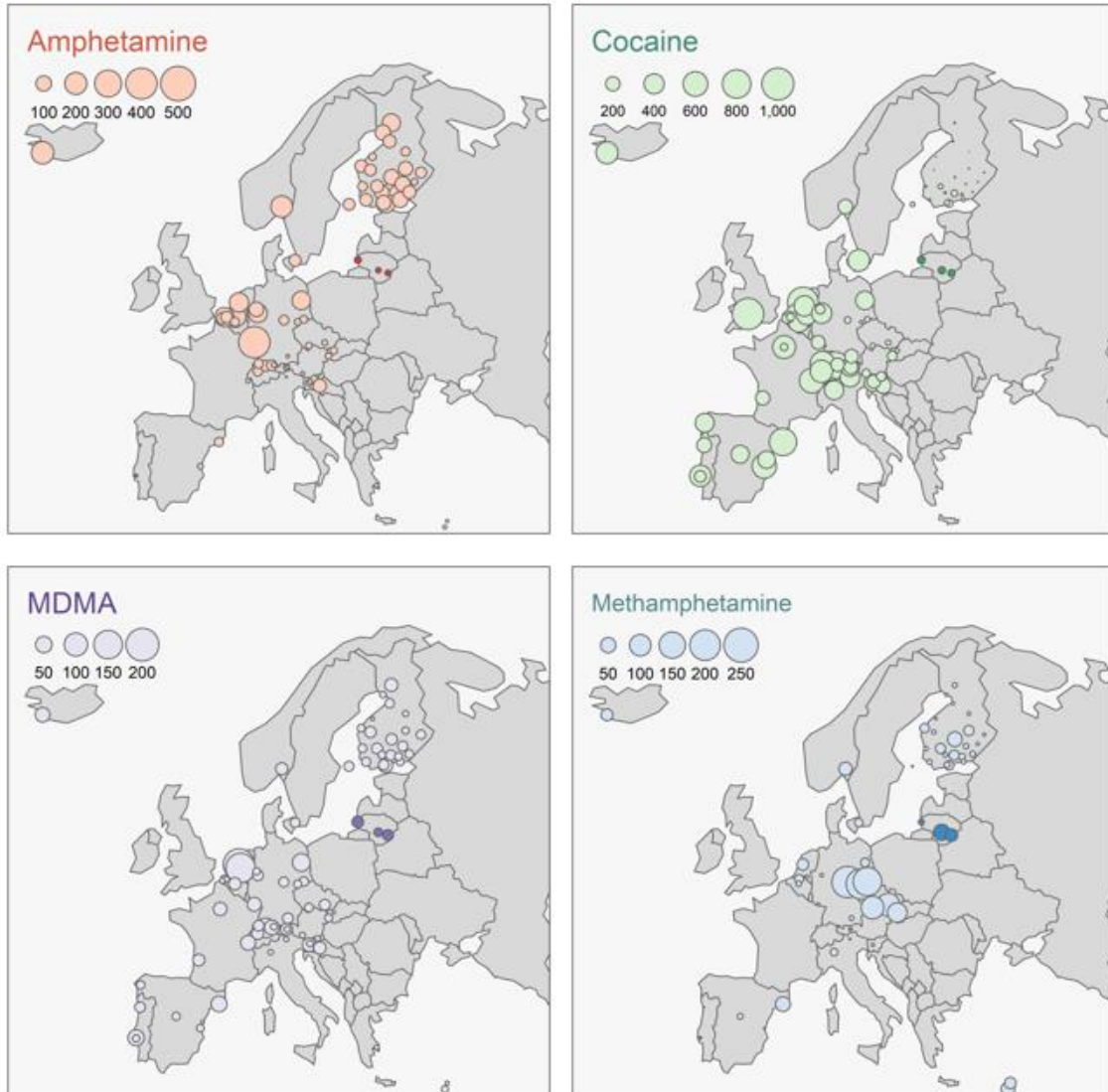
458

459 **Figure 1 Geographical map of Lithuania. Adapted from:** ⁵⁹



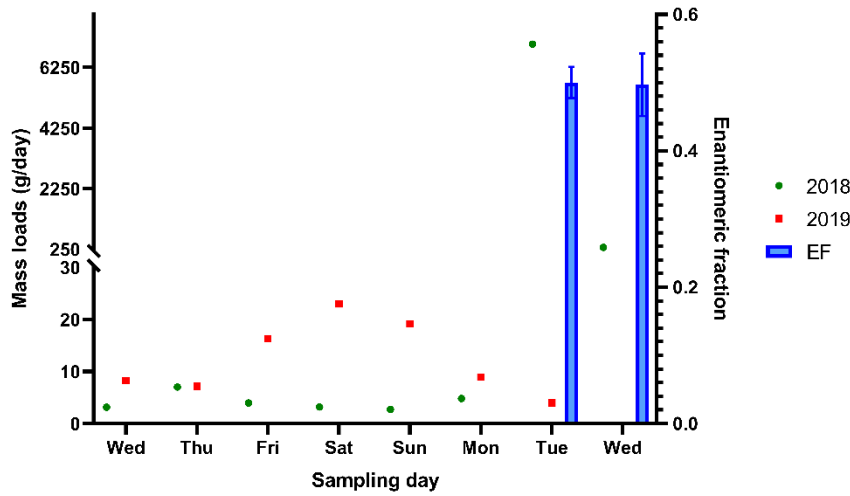
460

461 *Figure 2 Intra-country differences in stimulant consumption. The error bars in each location represent the daily variations*
 462 *in population-normalised mass loads. For figure 2B population-normalized mass loads were expressed for*
 463 *benzoylecgonine. Weighted averages were used to obtain national annual amounts of consumed stimulants.*



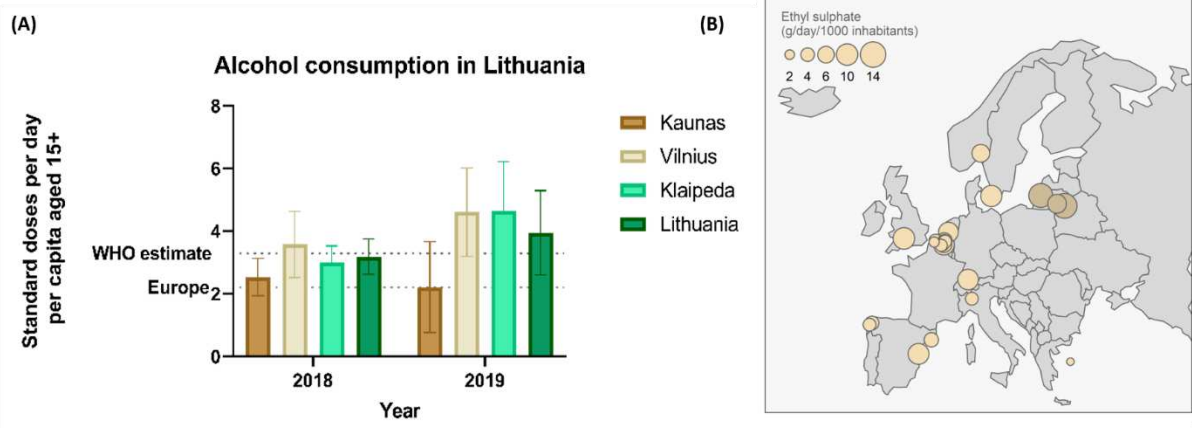
464
 465 **Figure 3** Population-normalized mass loads for AMP, BE, MDMA and METH (expressed as mg/day/1000 inhabitants) from
 466 2018. Mass loads from other countries were derived from the SCORE 2018 monitoring ³⁸. The deep coloured bubbles
 467 represent the Lithuanian cities.

Measured mass loads of amphetamine in Kaunas during the sampling week of 2018 and 2019



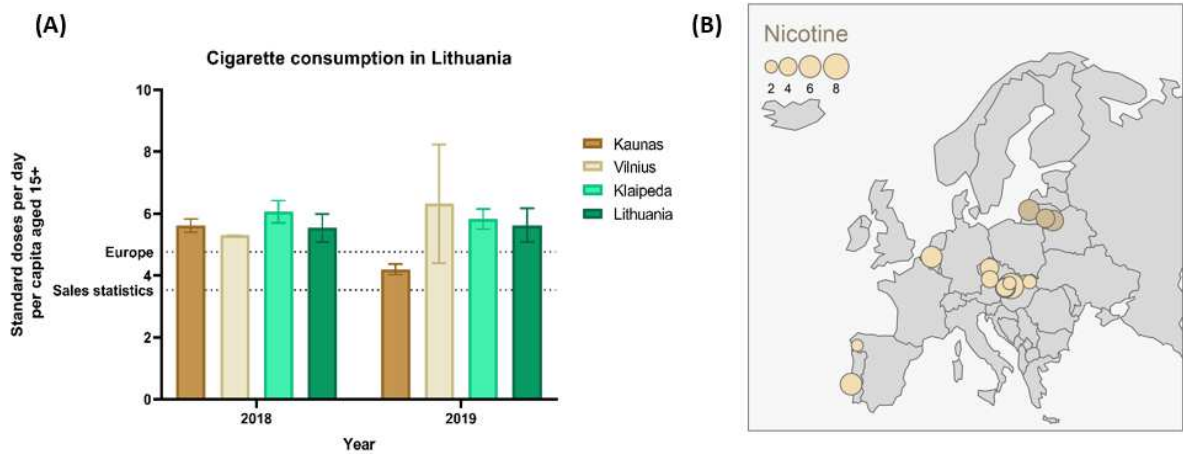
468

469 **Figure 4 Weekly variations in amphetamine loads (g/day) in Kaunas in 2018 and 2019.** Mass loads of amphetamine during
 470 2018 and 2019 in Kaunas were shown on the left Y-axis. Enantiomeric fractions (EF) for the influent wastewater samples of
 471 2018 were displayed on the right Y-axis. For the first six days of Kaunas in 2018 measured concentrations of (R)- and (S)-
 472 amphetamine were below the LLOQ during chiral analysis and therefore no EFs could be obtained for these days.
 473 Amphetamine measured on day 7 and day 8 was racemic.



474

475 **Figure 5 (A) Alcohol consumption (expressed as doses/day/capita aged 15+) in Lithuania in 2018 and 2019.** The Lithuania
 476 bar is the weighted mean of the three locations in that given year. The dotted lines are based on WHO estimates for
 477 Lithuania and Europe ⁴. (B) Comparison of population-normalized mass loads of ethyl sulphate between this study and
 478 other European WBE research



479

480 *Figure 6 (A) Cigarette consumption (expressed as doses/day/capita aged 15+) in Lithuania in 2018 and 2019. The*
 481 *Lithuania bar is the weighted mean of the three locations in that given year. The dotted lines are based on Tobacco Atlas*
 482 *estimates for Lithuania and Europe from 2016⁵⁰. The error bars represent the variation between daily population-*
 483 *normalised, COT and COT-OH derived mass loads. (B) Comparison of population-normalised loads of nicotine between*
 484 *this study and other European WBE research*

485

486

487 **Tables**488 **Table 1 Summary of sampling locations and periods**

Sampling location	Sampling period	Inhabitants served by wastewater treatment plant	% of Lithuanian population served by the WWTP	Age distribution (%)
Vilnius	12/04/2018-19/04/2018 11/09/2019-18/09/2019	536,631	19.2	0-14: 15-24: 25-44: 45-64: 65+:
Kaunas	11/04/2018-18/04/2018 27/03/2019-2/04/2019	288,363	10.5	0-14: 15-24: 25-44: 45-64: 65+:
Klaipeda	10/04/2018-16/04/2018 27/03/2019-02/04/2019	164,038	5.9	0-14: 15-24: 25-44: 45-64: 65+:

489

490 **Table 2 Leuckart-specific intermediates and impurities found in the IWW samples from Kaunas following the suspected**
491 **dumping event**

Name	Formula	Molecular weight (Da)	Theoretical molecular weight (Da)	Δppm	Retention time (min)	Peak intensity	Ratio (Dump)/(No dump)	Log-2 fold change (Dump)/(No dump)
Amphetamine	C9 H13 N	135.1047	135.104251	-3.5	9.02	4.02E+07	120	6.9
N-Ethylamphetamine	C11 H17 N	163.136	163.13555	-2.8	10.45	7.10E+06	106	6.7
N-Formylamphetamine (Formetorex)	C10 H13 N O	163.0996	163.0991656	-2.7	15.96	4.36E+08	1379	10.4
N-Formylmethamphetamine	C11 H15 N O	177.1152	177.1148156	-2.2	16.26	6.01E+07	240	7.9
4-Benzylpyrimidine	C11 H10 N2	170.0843	170.0838499	-2.5	16.32	1.45E+07	181	7.5
N,N-di-(b-phenylisopropyl)amine	C18 H23 N	253.1829	253.1825013	-1.4	17.24	2.28E+08	633	9.3
1-oxo-1-phenyl-2-(β-phenylisopropylimino)propane	C17 H19 N O	253.1466	253.1461158	-1.8	20.63	1.49E+08	283	8.2
N,N-di-(b-phenylisopropyl)formamide	C19 H23 N O	281.1781	281.1774159	-2.4	23.34	5.60E+07	45	5.5

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