

**Source identification of amphetamine-like stimulants in Spanish wastewater
through enantiomeric profiling**

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1 **Abstract**

2 Amphetamine (AMP), methamphetamine (MAMP) and 3,4-
3 methylenedioxyamphetamine (MDMA) occur in wastewater not only as a result of
4 illicit consumption, but also, in some cases, from prescription drug use or by direct drug
5 disposal into the sewage system. Enantiomeric profiling of these chiral drugs could give
6 more insight into the origin of their occurrence. In this manuscript, a new analytical
7 methodology for the enantiomeric analysis of amphetamine-like substances in wastewater
8 has been developed. The method consists of a solid-phase extraction (SPE) followed by
9 liquid chromatography-triple quadrupole-tandem mass spectrometry (LC-MS/MS),
10 which showed low quantification limits in the 2.4-5.5 ng L⁻¹ range. The LC-MS/MS
11 method was first applied to characterize a total of 38 solid street drug samples
12 anonymously provided by consumers. The results of these analysis showed that AMP and
13 MDMA trafficked into Spain are synthesized as racemate, while MAMP is exclusively
14 produced as the S(+)-enantiomer. Then, the analytical method was employed to analyse
15 urban wastewater samples collected from the wastewater treatment plants (WWTPs) of
16 five different cities in 2018 and 2019. Consumption estimated through normalized
17 population loads in wastewater showed an increased pattern of AMP use in the Basque
18 Country. Furthermore, the enantiomeric profiling of wastewater samples was contrasted
19 to lisdexamfetamine (LIS) and selegiline (SEL) prescription figures, two pharmaceuticals
20 which metabolize to S(+)-AMP, and to R(-)-AMP and R(-)-MAMP, respectively. From
21 this analysis, and considering uncertainties derived from metabolism and adherence to
22 treatment, it was concluded that LIS is a relevant source of AMP in those cases with low
23 wastewater loads, i.e. up to a maximum of 60% of AMP detected in wastewater in some
24 samples could originate from LIS prescription, while SEL does not represent a significant
25 source of AMP nor MAMP. Finally, removal efficiencies could be evaluated for the

26 WWTP (serving ca. 860,000 inhabitants) with higher AMP influent concentrations. The
27 removal of AMP was satisfactory with rates higher than 99%, whereas MDMA showed
28 an average removal of approximately 60%, accompanied by an enrichment of R(-)-
29 MDMA.

30

31 **Keywords:**

32 Drug abuse, chiral drugs, enantiomers, sewage, wastewater-based epidemiology,
33 prescription.

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39 1. INTRODUCTION

40 Amphetamine (AMP), methamphetamine (MAMP) and 3,4-
41 methylenedioxymethamphetamine (MDMA) are synthetic derivatives of
42 phenylethylamine that were used in the past to treat narcolepsy and spastic states of the
43 gastrointestinal tract (Myerson 1939). However, their stimulating effects have also been
44 associated to a high risk of addiction (Guttman and Sargent 1942, Lemere 1967).
45 Therefore, actions were taken to restrict their clandestine consumption.

46 Besides classical population surveys, hospital-related admissions and other
47 epidemiological indicators, efforts to detect illicit drugs' use in a fast and non-invasive
48 way led to the first study using wastewater-based epidemiology (WBE) in 2005 by
49 Zuccato et al. (Zuccato et al. 2005). They estimated cocaine consumption in a specific
50 population through the analysis of wastewater, as a complementary tool to the
51 established epidemiological approaches. Nowadays, this methodology has been applied
52 in many countries to get a near real-time profiling of the community-wide use of illicit
53 drugs (Bijlsma et al. 2021, González-Mariño et al. 2020, Ort et al. 2014, Thomas et al.
54 2012), alcohol and tobacco (Castiglioni et al. 2015, Gao et al. 2020, López-García et al.
55 2020, Montes et al. 2020, Rodríguez-Álvarez et al. 2015, Rodríguez-Álvarez et al. 2014a,
56 Rodríguez-Álvarez et al. 2014b, Ryu et al. 2016, Tschärke et al. 2016). Additionally,
57 WBE has been extended to estimate (unwanted) exposure to chemicals (Senta et al. 2020),
58 such as pesticides (Rousis et al. 2016), flame retardants (Been et al. 2018, Castro et al.
59 2020), bisphenol A (Lopardo et al. 2019) and plasticizers (Estévez-Danta et al. 2021,
60 González-Mariño et al. 2021, González-Mariño et al. 2017), and more recently as a useful
61 tool to follow and predict the evolution of COVID-19 (Ahmed et al. 2020, Alygizakis et
62 al. 2020, Medema et al. 2020).

63 A key factor in WBE studies is the selection of appropriate human biomarkers. However,
64 the estimation of AMP, MAMP and MDMA consumption is sometimes troublesome,
65 because the biomarkers usually measured in wastewater are the parent compounds (i.e.
66 unchanged excreted fraction), which can occur in wastewater not only as a result of illicit
67 consumption, but also from prescription drug use or direct disposal from waste of illegal
68 drug production (Emke et al. 2014). Yet, these three drugs are chiral and contain one
69 asymmetric carbon atom that leads to two enantiomers (R(-) and S(+)). In the human
70 body, this chirality implies different biological activity, and consequently, different
71 distribution and metabolism (Kalant 2001, Kasprzyk-Hordern et al. 2010).

72 Illicit AMP and MDMA are usually synthesized by the Leuckart method to yield a
73 racemic mixture (EMCDDA 2021a, b, Emke et al. 2018, Hauser et al. 2020, Kalant 2001,
74 King 2009), whereas MAMP is mainly produced as pure S-enantiomer across Europe,
75 with the only reported exception of Norway, where the synthesis facilities are different
76 than in Central Europe, and usually synthesize MAMP as a racemate (Castrignanò et al.
77 2018). AMP and MAMP are used in some countries as a prescribed medication to treat
78 attention deficit/hyperactivity disorder (ADHD), narcolepsy, or as a dietary supplement
79 to lose weight (Cody 2002). In Spain, AMP is not prescribed itself, but as the prodrug
80 lisdexamfetamine (LIS, used to treat ADHD), which is metabolized to S-(+)-AMP in the
81 human body (Comiran et al. 2021, Krishnan et al. 2008, Pennick 2013). MAMP and
82 MDMA do not currently have medical applications. However, selegiline (SEL), a
83 medication used in Parkinson treatment, metabolizes to produce the R(-)-enantiomer of
84 AMP and MAMP (Reynolds et al. 1978). Thus, both LIS and SEL could be potential
85 sources of AMP and MAMP in sewage, besides illicit consumption (Castrignanò et al.
86 2018, Lertxundi et al. 2021).

87 In the case of these three amphetamine-like substances, the S(+)-enantiomer is more
88 active and therefore metabolizes faster than the R(-)-enantiomer (Kasprzyk-Hordern
89 2010, Kasprzyk-Hordern and Baker 2012). This, consequently, results in a change of the
90 enantiomeric ratio towards the enrichment of the R(-)-enantiomer. Thus, mainly the R-
91 enantiomer is detected in untreated wastewater if the racemic drug is consumed. Hence,
92 enantiomeric analyses can complement traditional WBE estimates by applying analytical
93 methods that allow the determination of different chiral drug enantiomers, and therefore
94 differentiate between licit (prescription) or illicit use, or direct dumping in the sewage
95 network (Castrignanò et al. 2018, Emke et al. 2014, Gao et al. 2018).

96 Enantiomeric profiling has been mainly performed by liquid chromatography coupled to
97 tandem mass spectrometry (LC-MS/MS) in combination with a previous sample
98 concentration (usually a solid-phase extraction (SPE)). Kasprzyk-Horden and Baker
99 (Kasprzyk-Hordern and Baker 2012) were the first to address chiral analysis of these
100 substances. In that first study, Oasis MCX cartridges were used for SPE, due to the basic
101 nature of the illicit drugs studied. However, Oasis HLB cartridges have also been
102 employed in some other studies (Archer et al. 2018, Castrignanò et al. 2016, Vazquez-
103 Roig et al. 2014).

104 Although there are some WBE-derived studies targeting enantiomeric separation in
105 different countries (Archer et al. 2018, Castrignanò et al. 2018, Emke et al. 2014, Gao et
106 al. 2018, Kasprzyk-Hordern and Baker 2012), in Spain, enantiomeric profiling has been
107 applied only to two cities (Castellón and Valencia) of the Valencian Community region
108 (Castrignanò et al. 2018, Vazquez-Roig et al. 2014). Moreover, in a more recent study on
109 WBE of illicit drugs in Spain (Bijlsma et al. 2021) high levels of AMP were observed in
110 the area of Bilbao. However, the origin of such substance could not be fully clarified
111 (Lertxundi et al. 2021), even when a preliminary version of the enantiomeric profiling

112 method presented here was used. Hence, the aim of this work was to delve into spatial
113 differences by including 5 mid-to-large cities (and their metropolitan areas), located in
114 five Spanish regions and covering around 2 million people overall. To that end, we have
115 developed and validated a new enantiomeric analysis method and applied it to AMP,
116 MAMP and MDMA street drug samples obtained from different locations in Spain to
117 evaluate their enantiomeric fractions and purity. Also, wastewater samples from the
118 above-mentioned regions were collected in 2018 and 2019 and analysed. Finally, a
119 detailed discussion on the contribution of the prescription drugs LIS and SEL to the
120 amounts of AMP and MAMP detected in wastewater is presented for the first time.

121

2. MATERIAL AND METHODS

2.1 Chemicals and reagents

Individual solutions of 1 mg mL⁻¹ of AMP, MAMP and MDMA, and of 0.1 mg mL⁻¹ of their deuterated analogues (AMP-D₆, MAMP-D₅ and MDMA-D₅, used as internal standards (ISs)), were supplied by Cerilliant (Round Rock, TX, USA) as racemic mixtures. Individual solutions of 1 mg mL⁻¹ of the S-(+) enantiomer of AMP, MAMP and MDMA were supplied by Merck (Darmstadt, Germany).

Ultrapure water was obtained with a Millipore Milli-Q Gradient A-10 system (Bedford, MA, USA). LC-MS grade methanol (MeOH), formic acid (95-97%), ammonium bicarbonate (≥ 99.5%) and ammonia (NH₃) solution in water (25%) were supplied by Merck. Ammonia solution in MeOH (7N) was supplied by Across Organics (Thermo Fischer Scientific, Geel, Belgium).

2.2 Drug dose samples

Street drug samples were supplied by Energy Control and Ai Laket!! as powder or crystal. These two Spanish Organizations aim to reduce risks related to recreational drug use by providing fast and anonymous information to users on the composition of the drugs they are going to consume. Hence, such drugs were submitted to the harm-reduction, drug-checking services in an anonymous way and were then shipped to Santiago de Compostela for analysis. These drug samples were diluted to a nominal concentration of 250 ng mL⁻¹ of powder in MeOH, spiked with the ISs (100 ng mL⁻¹ each) and injected into the LC-MS/MS system.

2.3 Wastewater samples

Composite 24 h raw wastewater samples were collected at five wastewater treatment plants (WWTPs) located in Spain for 7 consecutive days in Spring 2018 and 2019, except

146 in the WWTP of Palma for which samples were only collected in 2018. Details on each
147 location, population served by each WWTP, and sampling are displayed in Table S1.

148 In addition, in 2019, treated wastewater samples from the WWTP of Galindo (Bilbao and
149 its large metropolitan area), in which high concentrations of AMP were detected, were
150 collected with a delay of 24 h with respect to raw wastewater (June 12th-18th) in order to
151 assess (enantioselective-)removal efficiencies, on request of the WWTP managers. This
152 WWTP treats the wastewater from over 850,000 inhabitants, with an average flow of ca.
153 250,000 m³ day⁻¹ (Table S1). The WWTP is equipped with a primary treatment
154 (flocculation and coagulation) and a secondary conventional activated sludge treatment,
155 including anoxic/anaerobic and aerobic treatments. The average hydraulic and sludge
156 retention times are 24 h and 22.5 days, respectively.

157 **2.4 Wastewater samples pretreatment**

158 Sample preparation was performed by two different analytical methods to identify and
159 quantify chiral drugs in wastewater.

160 *2.4.1 Method A*

161 All samples, except those from Castellón and Madrid (see 2.4.2) were processed in
162 Santiago de Compostela with method A, following the protocol described by González-
163 Mariño et al. (González-Mariño et al. 2018) with some modifications (see discussion on
164 3.2). Briefly, 100 mL of samples were vacuum-filtered through 0.7 µm GF/A glass
165 microfiber filters (Whatman, Kent, UK) and 0.45 µm cellulose acetate filters (Millipore)
166 and spiked with the ISs (100 ng L⁻¹ each). SPE was performed by mixed-mode reversed-
167 phase strong cation-exchange cartridges (Oasis MCX-150 mg, Waters, Milford, MA,
168 USA) previously rinsed with 5 mL of 5% NH₃ in MeOH followed by 5 mL of ultrapure
169 water. After loading, sorbents were dried under a nitrogen stream during 30 min, washed
170 with 4 mL of MeOH as a clean-up solution, and analytes were then eluted with 3 mL of

171 5% NH₃ in MeOH. Eluates were evaporated to dryness under a nitrogen stream using a
172 Turbo-Vap II (Zymark, Hopkinton MA, USA) and a Mini-Vap (Supelco, Steinheim,
173 Germany) concentrators. Finally, extracts were redissolved in 100 µL of MeOH, filtered
174 through 0.22 µm PVDF syringe-driven filters (Merck) and injected into the LC-MS
175 system.

176 *2.4.2 Method B*

177 Samples from Castellón and Madrid were extracted in the laboratory of the University
178 Jaume I, following the protocol described by Bijlsma et al. (Bijlsma et al. 2014a),
179 hereinafter, method B. In brief, 25 mL of sample were four-times diluted with ultrapure
180 water, spiked with 1 ng mL⁻¹ of ISs mixture and filtered through 0.45 µm cellulose filters
181 (Millipore). Then, SPE was performed by Oasis HLB-60 mg reversed-phase cartridges
182 (Waters) previously rinsed with 4 mL of MeOH followed by 4 mL of ultrapure water.
183 After loading, sorbents were dried under a nitrogen stream during 30 min and analytes
184 eluted with 5 mL of MeOH. Eluates were evaporated to dryness under nitrogen using a
185 Turbo-Vap II and a Mini-Vap concentrators. Finally, extracts were redissolved in 1 mL
186 of 10% MeOH in ultrapure water. These extracts were shipped frozen to Santiago de
187 Compostela, where they were evaporated to dryness, redissolved in 100 µL of MeOH and
188 filtered through 0.22 µm PVDF syringe-driven filters, being then ready for LC-MS/MS
189 analysis.

190 **2.5 Instrumental analysis**

191 Instrumental analysis was performed with a Waters Acquity UPLC[®] H-class system
192 equipped with a quaternary solvent pump, a thermostated LC column compartment, and
193 a sample manager. The UPLC system was interfaced to a triple quadrupole mass
194 spectrometer Xevo TQD from Waters.

195 The chromatographic separation was performed at 40 °C on a Lux AMP chiral column
196 (150×3 mm I.D., 3 µm particle size) from Phenomenex (Torrance, CA, USA). Under final
197 working conditions, a dual eluent system consisting of (A) ultrapure water with 50 mM
198 NH₃ and (B) MeOH was used at a flow rate of 0.4 mL min⁻¹. The linear gradient consisted
199 of the following stages: 0 min (60% B), 15 min (60% B), 20 min (95% B), 25 min (95%
200 B), 25.1 min (60% B) and 30 min (60% B). Injection volume was set at 10 µL.

201 The interface between the UPLC system and the Xevo TQD mass spectrometer was an
202 electrospray ionization (ESI) source operating in positive mode at a fixed capillary
203 voltage of 3 kV and a temperature of 150 °C. Nitrogen, provided by a nitrogen generator
204 from Peak Scientific (Barcelona, Spain), was used as desolvation gas at 600 L h⁻¹ and 450
205 °C, and as cone gas at 10 L h⁻¹. Analyses were performed by MS/MS in Selected Reaction
206 Monitoring (SRM) mode acquiring one precursor/product ion transitions per IS and two
207 transitions per analyte (one of them used for quantification and the second one for
208 confirmatory purposes). Argon was used as collision gas. Table S2 compiles chemical
209 formulae, retention times (RT), transitions (Q) and optimal cone voltages (CV) and
210 collision energies (CE) for every analyte.

211 **2.6 Method performance and quality assurance**

212 Instrumental detection and quantification limits (IDLs and IQLs) were estimated from the
213 lowest concentration level of the calibration curve providing a signal-to-noise ratio (S/N)
214 of 3 and 10, respectively. Calibration curves were prepared in MeOH and ranged from
215 the IQL to 2,500 ng mL⁻¹ for AMP enantiomers and from the IQL to 500 ng mL⁻¹ for the
216 enantiomers of the remaining compounds (spiked IS concentration, referred to the final
217 extract and each enantiomer: 100 ng mL⁻¹ for method A and 10 ng mL⁻¹ for method B).
218 Intra-day and inter-day instrumental precision were assessed by the relative standard
219 deviation (RSD %) of seven injections of two calibration standards, containing 5 ng mL⁻¹

220 ¹ and 50 ng mL⁻¹ of all analytes and 100 ng mL⁻¹ of IS. Injections were performed within
221 the same day (intra-day precision) and in four different days within a month (inter-day
222 precision).

223 Trueness and precision of the whole SPE-LC-MS/MS method A were assessed by
224 recovery studies in ultrapure water and wastewater spiked with 12.5 ng L⁻¹ and 125 ng L⁻¹
225 ¹, respectively, of all the analytes (100 ng L⁻¹ of IS). Wastewater aliquots spiked only with
226 ISs were also analysed to account for analyte levels in this matrix. Matrix effects (MEs)
227 were calculated as the signal (analyte peak area) percentage in a 125 ng mL⁻¹ spiked
228 wastewater extract, after non-spiked sample signal subtraction and referred to the signal
229 of a 125 ng mL⁻¹ standard. Method detection limits (MDLs) and method quantification
230 limits (MQLs) were calculated from non-spiked wastewater samples for a signal-to-noise
231 ratio (S/N) of 3 and 10, respectively.

232 In the case of method B, quality of data was assured by analysing additional extracts
233 provided by the University Jaume I, which had been previously already analysed in such
234 University and were used as quality controls (QC). Thus, four samples of wastewater
235 samples were spiked at two concentration levels (two of them with 50 ng L⁻¹ and the
236 remaining two with 400 ng L⁻¹, referring to each enantiomer) were extracted at the
237 University Jaume I, following sample pretreatment method B. The resulting extracts were
238 shipped to the University of Santiago de Compostela, where they were analysed to assess
239 method's trueness and precision, and to calculate the MDLs and MQLs (extrapolated
240 from the lowest level spiked sample).

241 **2.7 Calculations of enantiomeric fractions**

242 The elution order of enantiomers was confirmed by the analysis of S(+)-enantiomerically
243 pure standards. The concentration of each enantiomer was calculated by the internal

244 standard calibration method. Then, the concentration of each enantiomer (C_R for the R(-)
245)-enantiomer and C_S for S(+)-enantiomer) was used to obtain the enantiomeric fraction
246 (EF). In this work, EF is presented as EF_R , i.e. the ratio between the concentrations of the
247 R(-)-enantiomer and the sum of both enantiomers, as shown in Eq. 1.

$$248 \quad EF_R = \frac{C_R}{C_R + C_S} \quad Eq. 1$$

249 **2.7 Estimation of human illicit drugs consumption**

250 Drug concentration (sum of both enantiomers) in 24 h composite influent samples were
251 used to estimate population-normalized daily load levels (Eq. 2) of each drug and,
252 eventually, consumption (Eq. 3):

$$253 \quad \text{Daily loads} = \frac{\text{Concentration} \times \text{Flow rate}}{\text{Population}} \times 1000 \quad Eq. 2$$

$$254 \quad \text{Consumption} = \text{Daily loads} \times CF \quad Eq. 3$$

255 The correction factor (CF) values, which consider the fraction of drug excreted after
256 human metabolism, were: 2.77 (AMP), 2.3 (MAMP), and 4.4 (MDMA), as proposed in
257 (Gracia-Lor et al. 2016).

258

259 **3. RESULTS AND DISCUSSION**

260 **3.1 Liquid chromatography-tandem mass spectrometry**

261 Separation was performed on a Lux AMP column. A dual eluent system consisting of (A)
262 ultrapure water and (B) MeOH was used, with the addition of three different modifiers to
263 the aqueous phase: 5 mM of ammonium bicarbonate at pH 11 (recommended by the
264 column supplier), 50 mM of NH_3 at pH 11, and 5 mM of ammonium acetate at pH 9. An
265 adequate separation of enantiomers was observed at pH 11, independently of the modifier

266 used (ammonium bicarbonate and NH_3). Conversely, ammonium acetate at pH 9 could
267 not resolve the chromatographic peaks of the enantiomers, likely due to the incomplete
268 neutralization of the target species at this pH (Figure S1). Finally, 50 mM of NH_3 at pH
269 11 was selected as aqueous mobile phase additive due to the higher signal intensity and
270 lower noise observed as compared to the addition of ammonium bicarbonate (Figure S1).

271 Instrumental parameters investigated include linearity, IDLs, IQLs, and intra- and inter-
272 day precision (Table 1). The representation of the analyte area/IS area (response) versus
273 spiked analyte concentration (IQL-2,500 ng mL^{-1} range for each AMP enantiomer and
274 IQL-500 ng mL^{-1} range for the remaining enantiomers) fitted a linear model with
275 determination coefficients (R^2) higher than 0.997. IDL and IQL values varied between
276 0.2 ng mL^{-1} and 0.4 ng mL^{-1} , and between 0.6 ng mL^{-1} and 1.4 ng mL^{-1} , respectively.
277 RSD values from the intra-day precision varied between 0.7 % and 4.3 % for the 5 ng mL^{-1}
278 standard and between 0.5 % and 3.3 % for the 50 ng mL^{-1} level. RSD from the inter-
279 day precision was < 8.6 % at 50 ng mL^{-1} , and < 4.8 % at 5 ng mL^{-1} except for S(+)-
280 MAMP, for which it was 12%.

281 **3.2 Solid-phase extraction**

282 The extraction protocol applied in method A was based on a previous study (González-
283 Mariño et al., 2018) but modified in order to improve its selectivity, by including a clean-
284 up step, and optimizing the elution solvent volume. First, absolute recoveries for samples
285 extracted with Oasis MCX (125 ng L^{-1} spike level) were compared to the sample
286 preparation recoveries obtained when introducing a clean-up step with 4 mL of MeOH
287 before the elution (performed with 5% NH_3 in MeOH in both cases) (Figure S2a). Both
288 protocols showed good and comparable recoveries; thus, no significant losses were
289 observed due to the clean-up. Also, MEs were tested for both protocols, since previous
290 studies had reported improvements in this regard after the introduction of a clean-up step

291 (González-Mariño et al. 2012, González-Mariño et al. 2009, Senta et al. 2013).
292 Significantly lower matrix effects were observed (i.e. values of %ME close to 100%)
293 when the clean-up step was included (Figure S2b). Finally, the elution volume was
294 optimized by collecting three consecutive fractions of 3 mL of 5% NH₃ in MeOH, which
295 were analysed independently. More than 94 % of all analytes eluted in the first fraction
296 (data not shown), and, consequently, the elution volume was reduced from 10 mL in the
297 former method (González-Mariño et al., 2018), to only 3 mL.

298 **3.3 Method performance**

299 Method A was validated in terms of trueness, precision, MDLs and MQLs (Table 1).
300 Percentages of recovery (%R) for triplicate analyses of ultrapure water samples, spiked
301 with 12.5 ng L⁻¹ of all analytes and 100 ng L⁻¹ of IS, varied between 90% and 105%, with
302 RSDs between 1% and 6%. In raw wastewater samples spiked with 125 ng L⁻¹ of all
303 enantiomers and 100 ng L⁻¹ of IS, %R varied between 82% and 116 %, and RSD between
304 4% and 15%. MDLs ranged from 0.7 ng L⁻¹ to 1.8 ng L⁻¹, and MQLs from 2.4 ng L⁻¹ to
305 5.5 ng L⁻¹. Table S3 compares the performance of the proposed method versus other
306 analytical methods developed for the determination of chiral amphetamine-like
307 substances in raw wastewater. IQLs and MQLs were at the same order of magnitude than
308 those reported in other methodologies (Archer et al. 2018, Castrignanò et al. 2016,
309 Castrignanò et al. 2018, Gao et al. 2018, Kasprzyk-Hordern et al. 2010). However, all
310 those methods rely on the application of reversed-phase Oasis HLB cartridges, which can
311 perform well in terms of trueness (see also below), but do not offer the same degree of
312 selectivity as obtained by mixed-mode SPE. A further advantage of the method proposed
313 here is that the chromatographic separation is performed under gradient conditions, which
314 increases column lifetime when a complex matrix, as is the case of wastewater, is
315 analysed. Finally, the run time of the chromatographic method developed here is 30 min,

316 considerably lower than over 140 min required for other chiral separations (Kasprzyk-
317 Hordern 2010, Vazquez-Roig et al. 2014).

318 Since the samples from Castellón and Madrid had already been extracted by another SPE
319 protocol based on Oasis HLB cartridges (method B) and the enrichment increased by
320 further evaporation of the extracts (see 2.4.2), the performance of this protocol was
321 evaluated through recovery studies with the QC samples. As it is displayed in Table S4,
322 recoveries varied between 66 % and 125 % and RSD < 17 %; thus, this SPE protocol was
323 acceptable in terms of trueness and precision. MQL values, estimated from the lowest
324 concentration QC samples, ranged from 3.8 to 8.3 ng L⁻¹.

325 **3.4 Drug samples characterization**

326 Sample code, main drug, origin, purity and EF_R of each drug sample herein analysed are
327 listed in Table S5. The 38 drug dose samples were submitted to the two drug-checking
328 services (Energy Control and Ai Laket!!) by anonymous drug consumers. Consumers
329 labelled them as the drugs they expected them to contain: 18 were labelled as AMP, 6 as
330 MAMP, and 14 as MDMA. Sample purity (evaluated as explained in 2.2) is summarized
331 in Table 2 (details in Table S5). AMP samples presented a variable purity ranging from
332 2.7% to 103%. MAMP purity was more consistent and ranged between 54% and 76%,
333 while MDMA purity varied between 0% and 107%. No MDMA was found in the sample
334 coded MDMA-4, collected in Andalucía (Table S5). The results as regards EF_R show a
335 concordance with the reported synthesis route of these drugs in Europe (Castrignanò et
336 al. 2018, King 2009). Thus, AMP and MDMA samples were all racemate mixtures, while
337 MAMP samples were all the pure S(+)-enantiomer (Table 2 summarizes also the results
338 shown in Table S5). The limited number of samples does not allow us to address regional
339 patterns.

340 **3.5 Wastewater analysis**

341 EF_R of the amphetamine-like substances found in wastewater were calculated from the
342 concentrations measured in this matrix (see Eq. 1). Following WBE calculations, loads
343 (Eq. 2) and human consumption (Eq. 3) were subsequently estimated. The total
344 concentration (sum of the two enantiomers) measured is summarized in Table 3 (detailed
345 results are provided in Table S6). Excretion loads and estimated consumption values per
346 city, substance and year are summarized in Table 3 (further details in Tables S7 and S8).
347 Weekend peaks in loads/consumption (Tables S7 and S8) were observed in most locations
348 for MDMA, while this was not so clear in the case of AMP or MAMP. A potential
349 explanation is that these two last substances may originate either from daily abusers or
350 prescription patients, particularly in the case of AMP, as further discussed below. Similar
351 results have been observed in several other countries (Castrignanò et al. 2018, Thomas et
352 al. 2012).

353 *3.5.1 Amphetamine*

354 AMP was positively detected in all the wastewater samples. The high concentration levels
355 found in Bilbao and its metropolitan area in 2018 (mean 663 ng L⁻¹) (Bijlsma et al. 2021)
356 were confirmed in 2019 (mean: 1375 ng L⁻¹) (Table 3). Although considerably lower, the
357 second highest concentrations were detected in Palma (mean values: 106 ng L⁻¹, only
358 samples from 2018 available). The highest loads were observed in Bilbao in 2019 (mean
359 277 mg day⁻¹ 1000 inhabitant⁻¹), even higher than the loads reported in 2018 (mean 203
360 mg day⁻¹ 1000 inhabitant⁻¹). In the remaining cities, the estimated consumption of AMP
361 was lower than 45 mg day⁻¹ 1000 inhabitant⁻¹ (Table 3). These results match former
362 observations in Spain and confirms the distinct pattern of consumption in the area of
363 Bilbao, which is closer to the patterns observed in other countries such as Belgium,
364 Western Germany (Been et al. 2016) or some Nordic countries, where AMP is one of the
365 most prevalent drugs (González-Mariño et al. 2020).

366 The enantiomeric analysis showed a slight enrichment of R(-)-AMP in the wastewater of
367 most cities, with EF_R higher than 0.5 (Table 3, details in Table S9). This matches the data
368 obtained from the analysis of urine of 165 abusers with provided and average EF_R of
369 0.508 (George and Braithwaite 2000). This highlights the fact that enrichment of R(-)-
370 AMP (due to faster metabolization of S(+)-AMP) in the human body is not as high as in
371 the case of MDMA (see 3.5.3), therefore making it difficult to differentiate illicit
372 consumption from dumping events (that would lead to racemic AMP) on the basis of
373 enantiomeric analysis only, as described by Emke et al. (Emke et al. 2014). Despite this
374 fact, dumping it is unlikely to play a major role in our study since, even in the case of
375 Bilbao, where very high loads would point to direct disposal, no abnormal lead peak was
376 detected on any singular day and associated to a change in the value of EF_R (Tables S6-
377 S9), as already observed, for instance, for AMP and MDMA dumping events in The
378 Netherlands (Emke et al. 2014).

379 Yet, the EF_R obtained here are similar to those reported in other cities across Europe
380 (Castrignanò et al. 2018), including Valencia (Vazquez-Roig et al. 2014). In the case of
381 Castellón, the EF_R laid between 0.49 ± 0.03 and 0.50 ± 0.02 (Table 3), which is equivalent
382 to a racemic mixture. Samples from Castellón were also measured in 2015 by Castrignanò
383 et al. (Castrignanò et al. 2018), yet AMP was not detected that study. Conversely, an
384 enrichment of S(+)-AMP was detected in Madrid (Northern area) in 2018 with an average
385 EF_R of 0.41 ± 0.03 , while only the S(+) isomer was detected above the MQL in 2019 (Table
386 3). Such observation could be partly related to a high contribution of LIS prescription in
387 that area. In order to evaluate the potential contribution of medical prescription of LIS to
388 WBE-derived consumption estimations, these data were compared with the available LIS
389 prescription data from four of the five studied regions (Galicia, Basque Country, Balearic
390 Islands and Community of Madrid; data from the Community of Valencia was not

391 available). These data (as defined daily doses (DDD) $\text{day}^{-1}1000 \text{ inhabitants}^{-1}$) were
392 obtained on a month basis for the province or municipality (see details in Table S10).
393 Prescription data were converted into excretion loads of AMP, considering the DDD of
394 LIS (30 mg, https://www.whocc.no/atc_ddd_index/), the average excretion of S(+)-AMP
395 from LIS (44.75 %), and the molecular weights of both drugs. The excretion value of
396 44.75 % was derived as the average from two studies, performed with 7 individuals each,
397 where S(+)-AMP accounted for 48.5 % and 41 % of the LIS dose, respectively (Comiran
398 et al. 2021, Krishnan et al. 2008) (Table S11).

399 As it is displayed in Table 4, the expected loads of S(+)-AMP from LIS prescription range
400 from 1.0 to 7.0 $\text{mg day}^{-1} 1000 \text{ inhabitant}^{-1}$. When compared to the loads of AMP (sum of
401 both enantiomers) actually found in wastewater, the prescription of LIS would account
402 for less than 1% of the AMP consumption estimated in Bilbao and its metropolitan area,
403 clearly pointing to illicit drug use. Conversely, in Madrid (Northern area) about 58% of
404 AMP consumption in 2018 could be explained by prescription, which, together with EF_R
405 results (< 0.50 in 2018, R(+)-AMP below MQL in 2019) could confirm a mixed origin
406 (illicit use and LIS prescription). In Santiago de Compostela, medical prescription
407 contribution is expected to be relatively high (over 37-44%, Table 4), but the EF_R was
408 above 0.53 (Table S9) in all samples, which could then indicate that illicit consumption
409 would be more relevant than LIS prescription. This disagreement may be explained by
410 the fact that the external psychology consultations at the Santiago's hospital cover a larger
411 healthcare area, thus, many of the patients do not live in this area and thus do not contribute
412 to the wastewater samples. Further factors contributing to the uncertainty of the
413 estimations made are non-adherence to prescription, which has been calculated to be a
414 30% in Spain (Siffel et al. 2020). Therefore, data presented in Table 4 would likely
415 represent an overestimating scenario (maximum contribution of prescription) and the real

416 contribution of LIS would be lower. Even with these data in mind some EF_R values would
417 be higher than 0.5 in locations where prescription should be a relevant source. This is
418 further limited by the fact that when pure (George and Braithwaite 2000) or enriched
419 enantiomer (Cody et al. 2003) medications are prescribed, a certain degree of
420 interconversion occurs over time, which would lead to excretion of some R(-)-AMP and
421 not only pure S(+)-AMP.

422 Considering these limitations, it seems still evident that the contribution of LIS
423 prescription should be taken into account in future studies, particularly in those areas
424 where the amount of AMP measured in wastewater is rather low.

425 As regards the contribution of SEL prescription to R(-)-AMP in wastewater, the
426 expectable loads would range from 0.0028 to 0.022 mg day⁻¹ 1000 inhabitant⁻¹ (Table
427 S12). Those data were obtained from SEL prescription figures, considering a 15.4%
428 excretion rate, as the weighted average of 4 different studies with a total of 21 individuals
429 (detailed in Table S13) (Elsworth et al. 1978, Heinonen et al. 1989, Liebowitz et al. 1985,
430 Reynolds et al. 1978) and the corresponding molecular weights. Such loads can be
431 considered as negligible as they are two orders of magnitude lower than those from LIS,
432 therefore representing less than 0.2% of the total AMP in any of the WWTPs. Actually,
433 even in Palma, where the prescription of SEL was higher and with a dry precipitation
434 regime (thus lower WWTP inflows) such loads will translate into ca. 0.2 ng L⁻¹
435 concentrations of R(-)-AMP, i.e. below the MDL of the method.

436 *3.5.2 Methamphetamine*

437 Only S(+)-MAMP was detected in wastewater and the concentrations were low in all
438 cities, with average values below 45 ng L⁻¹ (Table 3) and maximum values up to 54 ng L⁻¹
439 (Table S6). S(+)-MAMP average loads were lower than 13 mg day⁻¹ 1000 inhabitants⁻¹

440 (Tables 3 and S7), confirming previous observations in Spain, with Barcelona, not
441 analysed here, being the exception (Bijlsma et al. 2021, González-Mariño et al. 2020).

442 The prescription of SEL would be equivalent to loads in the 0.0090-0.072 mg day⁻¹ 1000
443 inhabitant⁻¹ (Table S12), after considering an average excretion of R(-)-MAMP of 45.5%
444 from SEL, according to the metabolism data compiled in Table S13 (Elsworth et al. 1978,
445 Heinonen et al. 1989, Liebowitz et al. 1985, Reynolds et al. 1978). Again, as in the case
446 of AMP, considering Palma as the place where the highest contribution of SEL
447 prescription towards R(-)-MAMP is expected, this would result into concentrations of ca.
448 0.6 ng L⁻¹, which is below the MDL.

449 Thus, as regards the enantiomeric profiling of MAMP, EF_R was always 0, i.e. R(-)-
450 MAMP was below MDL in all samples (Table S9). These data reinforces the results
451 observed in 3.4 that indicated that MAMP consumed in Spain is synthesized as pure S(+)-
452 MAMP, as in most parts of Europe, while the contribution of SEL is negligible. This
453 would prevent from detecting any direct dumping event, but since no particularly high
454 concentrations could be detected, this was not expected to have occurred in any of the
455 WWTPs investigated during the sampling period.

456 3.5.3 MDMA

457 As for AMP, the two enantiomers of MDMA were detected in wastewater. The mean
458 MDMA concentration (as sum of both isomers) ranged from <MQL up to 374 ng L⁻¹
459 (Table S6). The highest concentrations were found in the wastewater from Palma, but
460 once corrected for population and flows, this does not translate into higher loads. Average
461 loads varied between 2-38 mg day⁻¹ 1000 inhabitant⁻¹ (Table 3), which is in the range of
462 the already estimated MDMA loads in Spain in former studies (González-Mariño et al.
463 2020).

464 The EFR average for MDMA was 0.61 ± 0.05 , being above 0.5 in all samples (Table S9)
465 thus indicating the predominance of R(-)-MDMA in wastewater. These values match with
466 urinary data, where 6 volunteers administered 100 mg of racemic MDMA led to an
467 average EFR of 0.657 over 24 h (Pizarro et al. 2002). Furthermore, an autopsy study
468 revealed an EFR of 0.57 (Moore et al. 1996). As MDMA is trafficked as racemate (see
469 3.4) and the S(+)-enantiomer is metabolized faster in the human body, the observed
470 enrichment of R(-)-MDMA corroborates illicit consumption as the main source of
471 MDMA in wastewater (Castrignanò et al. 2018). No event of drug disposal in the sewage
472 network was detected.

473 *3.5.4 Treated wastewater*

474 Given the high concentrations of AMP detected in the WWTP of Bilbao and its
475 metropolitan area in both 2018 and 2019, treated wastewater samples were also collected
476 in 2019 and in the same way as raw wastewater, but with a delay of 24 h to account for
477 the hydraulic residence time in the plant. AMP and MAMP levels were below the MQL
478 in all treated wastewater samples, whereas MDMA was detected in all samples. As
479 detailed in Table S14, MDMA levels ranged from 31 to 99 ng L^{-1} (average 57 ng L^{-1}),
480 which is similar to the median value of 56 ng L^{-1} measured in the effluents of 42 WWTPs
481 from the region of Catalonia (Spain) collected during 2006 and 2007 (Huerta-Fontela et
482 al. 2008). Little is known about the ecotoxicological effects of MDMA, but the
483 anticipated value of predicted non-effect concentration for this substance is 220 ng L^{-1}
484 (Fernández-Rubio et al. 2019), hence emissions from the WWTP are not expected to
485 generate ecotoxicological effects. Yet, there is a clear need for further experimental data,
486 particularly considering the co-occurrence of different enantiomers (Sanganyado et al.
487 2017).

488 These data imply that the removal of AMP in the WWTP, considering the MQL, was
489 higher than 99%, while MDMA nominal removal was 61% when considering the average
490 concentrations measured in the effluent and influent (MAMP was below the MQL in both
491 types of wastewater). Similar good removal rates for AMP but a higher variability in the
492 case of MDMA have been reported in the literature (Bijlsma et al. 2012, Bijlsma et al.
493 2014b, Huerta-Fontela et al. 2008, Yadav et al. 2017).

494 Furthermore, the EF_R of MDMA shifted from 0.68 ± 0.07 in the influent to 0.88 ± 0.04 in
495 the effluent, which implies a further prevalence of the less biologically active enantiomer,
496 R(-)-MDMA, after the wastewater treatment and a clear distinct elimination profile of
497 both enantiomers, i.e.: average removals of 21% and 77%, for R(-)-MDMA and S(+)-
498 MDMA, respectively. Such enantioselective elimination has already been observed in a
499 WWTP in Valencia studied in 2012 (Vazquez-Roig et al. 2014) but, as mentioned,
500 ecotoxicological implications remain unknown.

501

502 **4. CONCLUSIONS**

503 An analytical methodology based on SPE and LC-MS/MS has been successfully
504 developed for the determination of three chiral amphetamine-like substances in urban
505 wastewater and street drug samples. The analyses of consumer-donated street drugs
506 clearly indicated that AMP and MDMA are produced as racemic mixtures for the Spanish
507 illicit market, while MAMP is produced as the pure active S(+)-enantiomer. The
508 enantiomeric profiling from wastewater analyses indicated that much higher levels of
509 AMP occur in the metropolitan area of Bilbao compared to other Spanish cities, which,
510 combined with LIS and SEL prescription data indicate that its origin can be attributed
511 mainly to illicit consumption. Conversely, in the remaining Spanish cities investigated,
512 where AMP levels are low, the prescription of LIS may become a relevant source of AMP,

513 whereas the contribution of the prescription of the pharmaceutical SEL to AMP and
514 MAMP loads is negligible. Finally, the analysis of effluent samples in the area of Bilbao
515 showed that AMP is well removed in a WWTP equipped with conventional biological
516 water treatments, while the removal of MDMA is relatively high for the S(+)-enantiomer,
517 but much more limited for R(-)-MDMA.

518

519 **CRedit authorship contribution statement**

520 **Andrea Estévez-Danta:** Investigation, Methodology, Formal analysis, Visualization,
521 Writing - original draft. **Rosa Montes:** Methodology, Supervision, Formal analysis,
522 Writing - original draft. **Lubertus Bijlsma:** Investigation, Resources, Methodology,
523 Writing - review & editing. **Rafael Cela:** Resources, Funding acquisition. **Alberto**
524 **Celma:** Investigation, Resources, Writing - review & editing. **Iria González-Mariño:**
525 Methodology, Supervision, Writing - review & editing. **Manuel Miró:** Resources,
526 Writing - review & editing. **Vanessa Gutmann:** Investigation, Methodology. **Unai**
527 **Pérez de San Román-Landa:** Resources. **Ailette Prieto:** Resources, Writing - review
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529 Resources, Supervision, Funding acquisition, Formal analysis, Writing - review &
530 editing. **José Benito Quintana:** Resources, Visualization, Supervision, Funding
531 acquisition, Writing - review & editing.

532

533 **Declaration of competing interest**

534 The authors declare that they have no known competing financial interests or personal
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558

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