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7  
8 **Analysis of chiral pharmaceutical residues in influent and effluent samples at racemic**  
9 **and enantiomeric level using liquid chromatography-tandem mass spectrometry**

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15 **Highlights:**

- 16 • Development of method for chiral pharmaceuticals determination.  
17 • Greenness assessment of developed and validated method.  
18 • d-SPE extraction of chiral compounds from wastewater samples.  
19 • Seasonal monitoring of selected pollutants in wastewater at enantiomeric level.

20 **Abstract:**

21 In this work, two different chromatographic methods for seasonal monitoring of  
22 pharmaceutical residue in wastewater samples were developed. In the case of enantiomeric  
23 separation of selected compounds, LC-MS technique combining with vancomycin based chiral  
24 stationary phase was used. The performance of chiral analysis enabled to monitor the  
25 pharmaceutical contamination at the enantiomeric level. The d-SPE procedure was developed as  
26 sample preparation step and compared with SPE protocol in terms of recoveries and environmental  
27 friendliness. Due to satisfactory recoveries (around 60%) and greener character assessed using

28 GAPI and AGREE tools, d-SPE-LC-MS/MS method was applied in further analysis. The  
29 concentration of detected enantiomers in wastewater collected in different seasons did not exceed  
30  $10 \mu\text{g L}^{-1}$ , whereas the evaluated EF values were generally in the range of 0.4-0.7. Moreover, no  
31 significant changes in EF values after wastewater treatment were observed.

32 **Keywords:** Chiral analysis; GAPI; AGREE; wastewater; dispersive solid phase extraction; chiral  
33 pharmaceuticals

## 34 1. Introduction

35 The monitoring of pharmaceutical residues in effluent can be considered as an indicator  
36 of the total pollution of the aquatic ecosystem. The sources of human pharmaceuticals in  
37 wastewater are mainly based on hospitals, health-care facilities and households, while the animal  
38 origin pharmaceutical sources are mostly from husbandry including those for non-therapeutic  
39 purposes. The amount of drugs observed can range from  $\text{ng L}^{-1}$  to  $\mu\text{g L}^{-1}$  levels [1–3] with regard  
40 to season, geographical location and local administration practices. Due to lipophilic character of  
41 some of these pollutants, they may enter the food-chain and accumulate in the fat tissues of aquatic  
42 organisms. The effects of presence of pharmaceuticals in the environment are different, but they  
43 may cause zooplankton and phytoplankton extinction, feminization of male individuals, bacterial  
44 resistance to antibiotics, and in fish, even kidney and bronchial damage [3,4]. Due to the water  
45 cycle and migration, many of them can be quantified in tap water and groundwater [5,6].

46 The National Association of Clean Water Agencies report [7] states that even if the  
47 observed quantities of pharmaceuticals in various water sources are 1000 smaller than the toxic  
48 levels, the accumulative effect on the exposed group (children, the elderly) draws attention. Even  
49 in advanced wastewater treatment plant (WWTP), where ozone and UV-assisted treatment methods  
50 are used, the pharmaceutical residues in the effluent are still at detectable levels [8–10]. Currently,  
51 there is no ecotoxicity prevention in this area, but it may be concluded that increasing use of  
52 pharmaceuticals will be the foremost environmental and human pollutants in the coming future. So  
53 far, there is no legal obligation applied for pharmaceutical residues elimination in wastewater  
54 treatment processes, but the contamination of drinking water, vegetables, meat, fishes and seafood  
55 or dairy products is a fact with growing attention while the effect of long-term exposure of  
56 pharmaceuticals to humans are not clarified by the researchers [3].

57 The impact of pharmaceuticals on urban pollutant load must be considered on  
58 enantiomeric levels, due to the fact that approximately 80% of the drugs are sold as a racemate  
59 mixture. The enantiomers of these racemic mixtures exhibit different physiological and  
60 toxicological effects [11]. Therefore, enantiomeric determination of pharmaceuticals in wastewater  
61 will provide important information on both the level of environmental toxicity and the  
62 concentration of pharmaceuticals that may reach human beings again [12]. However, due to the  
63 latest trends, the procedure for enantiomeric determination should be developed in accordance with



64 the principles of Green Analytical Chemistry (GAC). The aim of GAC is to reduce the impact of  
65 analytical procedures on the environment. One of the most common mode of GAC application is  
66 the reduction of the extraction steps in the sample preparation. Moreover, it is proposed to skip  
67 some sample preparation steps, apply direct analysis, and to use eco-friendly mobile phases. In  
68 case of chiral analysis, the environmental friendly approaches should be given more attention,  
69 as the chromatographic run is mainly performed with high amounts of toxic solvents. Besides,  
70 the elimination of the derivatization step of the analytes can be qualified as green approach as well  
71 [13].

72 The aim of this study is to develop an analytical approach to the determination of chiral  
73 pharmaceuticals in wastewater samples and assess its greenness. Firstly, a reversed- phase liquid  
74 chromatography coupled with tandem mass spectrometry (RP-LC-MS/MS) was used to develop a  
75 method for the monitoring of presence of selected pharmaceuticals in influent and effluent samples.  
76 Secondly, a chiral-LC-MS/MS method was developed and validated to determine the enantiomers  
77 of the selected pharmaceutical residues together with enantiomeric factor calculation. Moreover,  
78 sample preparation step was carried out using two different types of extraction; solid phase  
79 extraction (SPE) and dispersive solid phase extraction (d-SPE). The development of d-SPE  
80 protocol enabled to reduce the solvent consumption, time needed for extraction and the labor.

## 81 2. Experimental

### 82 2.1. Analytes Selection

83 The selection of pharmaceuticals for analysis varied depending on the types, uses, seasons,  
84 and demographic structure in which samples were collected. The amount of drugs unchanged  
85 excreted should be at very low levels in large volume of wastewater, therefore, it was decided that  
86 frequently used pharmaceuticals would be subjected to this analysis. The six pharmaceuticals  
87 selected for these studies; atenolol (ATE), fluoxetine (FLX), ibuprofen (IBU), ketoprofen (KET),  
88 omeprazole (OME) and ofloxacin (OFL), are characterized in the **Supplementary Material,**  
89 **Table S1.** These pharmaceuticals belong to different groups of drugs, such as  $\beta$ -blockers, serotonin  
90 reuptake inhibitors, non-steroidal anti-inflammatory drugs (NSAID), proton pumps and  
91 antibiotics, which were believed to have a high frequency of occurrence in wastewater samples.

### 92 2.2. Chemicals and Materials

93 All standards were of the analytical purity and commercially available. The standards of  
94 pharmaceuticals, all in racemic form, were purchased from Sigma-Aldrich (St. Louis, USA).  
95 Ammonium formate, formic acid and methanol were all HPLC and bought from Merck (Darmstadt,  
96 Germany). The ultrapure water was prepared using HPL5 system from Hydrolab (Wiślina, Poland).  
97 The cartridges used for SPE were Strata-X Polymeric RP (200 mg, 3 mL) purchased from  
98 Phenomenex (Torrance, USA), Oasis HLB (200 mg, 6 mL) obtained from Waters Corporation  
99 (Milford, USA) and Lichrolut NH2 (200 mg/3 mL) purchased from Merck (Darmstadt, Germany).  
100 The d-SPE sorbents used were made of reverse-phased polymeric sorbent, silica gel modified with  
101 octadecyl group and an amino-modified silica gel, obtained from Phenomenex (Torrance, USA),  
102 Macherey-Nagel (Dueren, Germany) and Merck (Darmstadt, Germany) respectively.

103 The buffer solution was prepared by dissolving the required amount of ammonium  
104 formate in water and pH was maintained to 3.6 with formic acid. Mobile phase for  
105 Chiral-LC-MS/MS was prepared by dissolving required amount of ammonium formate in methanol  
106 with 0.005% formic acid.

107 Stock solutions of pharmaceuticals were prepared in methanol. All solutions were kept in  
108 4°C while prepared samples were stored in -20°C until the analysis. Sodium N-methylcyclohexyl  
109 sulfate was used as the internal standard (IS).



## 110 2.2. Instruments and Analytical Conditions

111 All analyses were performed using a liquid chromatograph (Nexera X2, Shimadzu, Japan)  
112 coupled with triple quadrupole mass spectrometer (LCMS 8060, Shimadzu, Japan) equipped with  
113 an electrospray ionization (ESI) source operating in positive mode for ATE, FLX, OME, OFL and  
114 IBU and negative mode for KET and IS. The multiple reaction monitoring mode (MRM) was  
115 chosen for qualitative and quantitative analysis. The optimization of MRM conditions were  
116 performed using 100 ng mL<sup>-1</sup> solutions of each analyte and the LC-MS system was set to work in  
117 the flow injection analysis mode (FIA). The direct injection of individual standard solutions of each  
118 analyte allowed to choose the compound precursor ion. Then, each precursor ion was fragmented  
119 in the collision cell to obtain specific product ions. Two the most intense ions were chosen as the  
120 MRM transitions for analytes. The optimized parameters of MS/MS mode are presented in  
121 Table 1.

122 **Table 1.** Parameters of the monitored ion transitions

Analyte	Precursor ion [m/z]	Product ions	Collision Energy [V]	Q1 Prerod [V]	Q3 Prerod [V]
ATE	267.00	145.20	-25	-13	-30
		190.20	-18	-13	-12
FLX	310.10	44.16	-11	-10	-18
		148.30	-8	-14	-15
IBU	207.10	45.20	-20	-10	-18
		89.10	-12	-16	-20
KET	253.10	209.10	9	17	21
OME	346.00	198.15	-12	-20	-20
		151.25	-20	-12	-14
OFL	362.10	318.20	-20	-12	-21
		261.15	-27	-10	-12
IS	192.20	79.90	29	14	29

123 The parameters for capillary voltage (4 kV), drying gas flow (10 L min<sup>-1</sup>); nebulizing gas  
124 flow (3 L min<sup>-1</sup>), interface temperature (300°C), desolvation line temperature (250°C) and heat  
125 block temperature (450°C) were optimized by injecting a mixture of the analytes. The LabSolutions  
126 Software was used for data acquisition.



127 ACE Ultracore 2.5 SuperC18 (100 x 2.1 mm, 2.5  $\mu\text{m}$ ) was chosen for the analysis in  
128 RP mode. The column temperature was kept at 45°C. The flow rate was 0.7 mL min<sup>-1</sup> and  
129 the injection volume was 2  $\mu\text{L}$  all through the analysis. The mobile phase used for the separation  
130 consisted of 25 mM ammonium formate (pH 3.6. using formic acid) (Component A) and methanol  
131 (Component B). The gradient elution used for the chromatographic separation was as follows:  
132 10% B in 0 min, 10% B in 1 min, 95% B in 8 min, 95% B in 10 min. After each analysis the initial  
133 conditions were restored in 5 min.

134 Chiral-LC-MS/MS analysis were performed using Astec Chirobiotic V column  
135 (150 x 4.6 mm). The column temperature was kept at 25 °C, the flow rate was 0.5 mL min<sup>-1</sup> and  
136 the injection volume was 10  $\mu\text{L}$  all through the analysis. The mobile phase used for the separation  
137 was consist of 4 mM ammonium acetate and 0.005% formic acid in methanol.

### 138 **2.3. Calibration Solutions and Validation Formulas**

139 Six-point calibration curves were prepared and analyzed several times (n = 3). Calibration  
140 solutions were prepared in MeOH in range of 0.5-25 ng mL<sup>-1</sup>. For each solution, the same amount  
141 of IS was added (10  $\mu\text{L}$ ). Calibration curves were constructed using the internal standard method,  
142 where the ratio of analyte peak area to IS peak area was taken under the consideration. The values  
143 of limit of detection (LOD) and limit of quantification (LOQ) were calculated from the following  
144 equations:  $LOD = 3.3 \times S_b/a$  and  $LOQ = 3 \times LOD$ , where  $S_b$  is standard deviation of the intercept of  
145 the calibration curve, and  $a$  is a slope of the calibration curve. For method validation, standard  
146 solutions were prepared at three levels (1, 5 and 10 ng mL<sup>-1</sup>). These samples were used for  
147 evaluation of the accuracy and precision of the developed procedure. One series of standard sample  
148 (5 ng mL<sup>-1</sup>, n= 5) was analysed for the next three days to determine the repeatability.

### 149 **2.4. Sample Collection and Preparation**

150 Average daily influent (INF) and effluent (EFF) samples were collected in winter, spring,  
151 summer and autumn from urban WWTP located in Northern Poland (Pomeranian Voivodeship).  
152 This WWTP is using mechanical, chemical, and activated biological treatment, and purify about  
153 55 000 m<sup>3</sup> sewage per day. This place is surrounded by tourist cities and villages located nearby



154 the Baltic Sea and received mainly domestic and industrial discharges, especially from food  
155 industry (e.g., fish processing).

156 Collected wastewater samples were stored in amber glass bottles in a refrigerator at 4°C  
157 until extraction (no longer than 48 hours). In case of SPE, 50 mL of influent or effluent samples  
158 were passed through the conditioned cartridges, after which the cartridges were dried for  
159 20 minutes under vacuum. Next, the analytes were eluted by methanol, mixture of methanol,  
160 acetone and ethyl acetate and finally by ammonia solution (2:1:1 v/v). The excess of solvent was  
161 removed to dryness under a gentle stream of nitrogen at 45°C. Finally, the residues were dissolved  
162 in 1 mL of methanol. In case of d-SPE, 200 mg of sorbent was added to centrifuge tubes with  
163 45 mL of filtered influent or effluent sample. The samples were shaken for 45 minutes and then  
164 centrifuged for 10 minutes. The supernatants were removed and the extraction solvents were added.  
165 Then, the extracts were filtrated in order to remove the sorbent from the samples and later  
166 evaporated to dryness. The dried extracts were dissolved in 1mL of methanol.

### 167 **3. Results and Discussion**

#### 168 **3.1. Separation of Analytes Using RP-LC-MS/MS Mode and Polar Organic Chiral** 169 **Mode**

170 In case of this study, a series of experiments was performed in order to obtain separation  
171 of six analytes, short analysis time (less than 10 minutes), as well sensitivity and reproducibility  
172 needed to determine analytes in the samples. The column with narrow diameter (2.1 mm) was  
173 chosen to reduce the amount of mobile phase used. All analyses were performed in the gradient  
174 elution mode, which was optimized together with the temperature of separation. A buffer with pH  
175 3.6 was chosen due to improved peak shape and resolution, whereas methanol was chosen as an  
176 organic component of mobile phase. Suspecting that the test analytes in the samples are in form of  
177 racemates, the chiral separation was performed. The Chirobiotic V column bed is based on bonding  
178 vancomycin, so only basic molecules such as FLX and ATE were selected for further optimization.  
179 Due to highly polar character of FLX and ATE, long analysis time was expected. As it is performed  
180 in the literature [2,14,15], buffers with ammonium salts (formate, acetate) are effectively used in  
181 enantiomeric separation. Hence, two different mobile phase compositions were tested. The first  
182 one consisted of methanol and ammonium acetate buffer (9:10 v/v), whereas the second one





183 consisted of 99.95% methanol with 4 mM ammonium acetate with addition of 0.005% of formic  
184 acid. The addition of formic acid caused enantiomer peaks to be well resolved from baseline  
185 ( $R_s > 1.5$ ). Higher flow rates were also tested in order to minimize the analysis time but the  
186 resolution between ATE enantiomers decreased ( $R_s < 1.0$ ).

### 187 3.2. Chiral-LC-MS/MS Method Validation

188 The method dedicated to chiral analytes was validated according to the guidelines for  
189 analytical method validation [16]. The parameters such as linearity, LOD, LOQ, recoveries and  
190 repeatability were studied. The results from validation are presented in Table 2 and 3. All  
191 constructed calibration curves were linear in the analysed concentration range (0.5-25 ng mL<sup>-1</sup>),  
192 with  $R^2$  above 0.997 and LOD below 0.1 ng mL<sup>-1</sup>. Due to the strict connection between obtained  
193 results and the developed method, it is recommended to calculate LOD values based on the  
194 calibration curve. The recoveries obtained for *S*-ATE, *R*-ATE, *S*-FLX and *R*-FLX were around  
195 100%. Hence, the obtained results are satisfactory in terms of accuracy, precision and repeatability.  
196 Therefore, the developed method is suitable for determining the enantiomers of ATE and FLX in  
197 the INF and EFF samples.

198 **Table 2.** Data gathered from equations of calibration curves.

Analyte	Calibration Curve Equation	$S_a$	$S_b$	LOD [ng mL <sup>-1</sup> ]	LOQ [ng mL <sup>-1</sup> ]	$R^2$
<i>S</i> -ATE	$y = 0.8641x - 0.018$	0.0067	0.014	0.051	0.15	0.9991
<i>R</i> -ATE	$y = 0.9013x - 0.020$	0.0063	0.012	0.045	0.13	0.9989
<i>S</i> -FLX	$y = 0.1335x + 0.0030$	0.0015	0.0028	0.070	0.21	0.9982
<i>R</i> -FLX	$y = 0.1335x + 0.0026$	0.0019	0.0036	0.088	0.27	0.9976

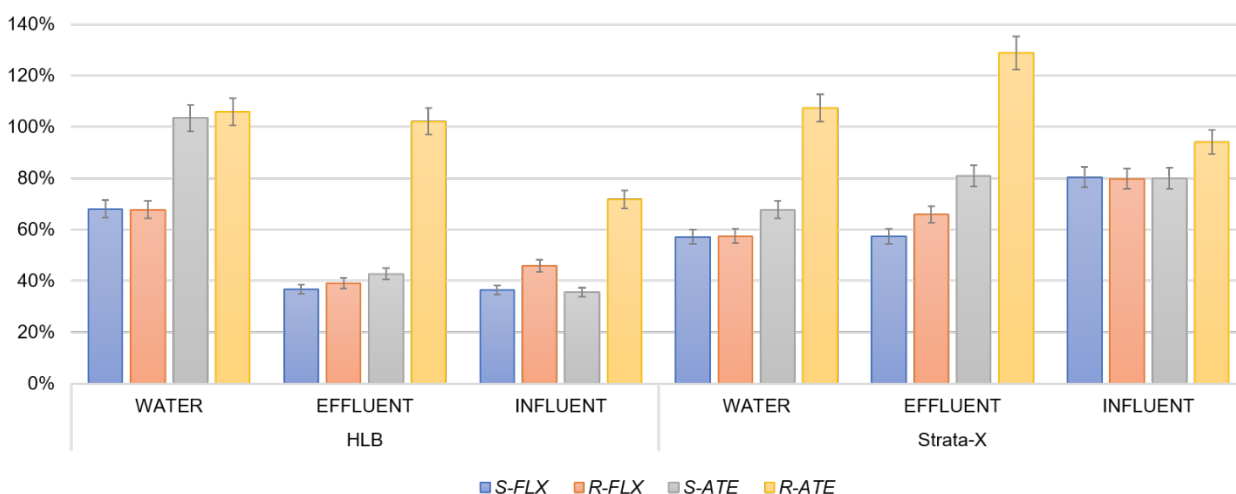
199

200 **Table 3.** Accuracy, precision and recovery values obtained for the studied compounds.

Analyte	Spiking level [ng mL <sup>-1</sup> ]	Mean recovery [ng mL <sup>-1</sup> ] (%); n=5	SD [ng mL <sup>-1</sup> ]	CV [%]	Repeatability, n=5			
					Day	Mean recovery [ng mL <sup>-1</sup> ] (%)	SD [ng mL <sup>-1</sup> ]	CV [%]
<i>S</i> -ATE	1	1.003 (100)	0.025	2.5	1	4.81 (96)	0.12	4.2
	5	4.81 (96)	0.12	4.2	2	5.00 (100)	0.11	2.2
	10	9.89 (99)	0.39	3.9	3	4.95 (99)	0.13	2.7
<i>R</i> -ATE	1	1.040 (104)	0.022	2.1	1	5.02 (100)	0.20	4.0
	5	5.02 (100)	0.20	4.0	2	5.25 (104)	0.10	1.9
	10	10.04 (100)	0.27	2.7	3	5.20 (104)	0.16	3.2
<i>S</i> -FLX	1	1.044 (104)	0.021	2.0	1	5.08 (102)	0.22	4.3
	5	5.08 (102)	0.22	4.3	2	5.40 (108)	0.21	3.8
	10	11.52 (115)	0.54	4.7	3	5.14 (103)	0.23	4.3
<i>R</i> -FLX	1	1.051 (105)	0.018	1.7	1	4.93 (99)	0.21	4.2
	5	4.93 (99)	0.21	4.2	2	5.25 (105)	0.10	2.0
	10	11.33 (113)	0.50	2.7	3	5.19 (104)	0.23	4.4

201  
 202 **3.3. Optimization of Sample Preparation Step**  
 203 In order to get the highest recoveries of **ATE** and **FLX**, different SPE and d-SPE  
 204 approaches were used. The choice of the best sorbent that gives an acceptable recovery for analytes  
 205 with different physicochemical properties plays a crucial role in method development applied in  
 206 SPE. In first step, the polarity of FLX and ATE was determined using the ALOGPs 2.1 program.  
 207 This algorithm enables to calculate the octanol/water coefficient (log P) on the basis of SMILE  
 208 structure. The results for FLX and ATE are presented in **Supplementary Materials, Table S2**.  
 209 ATE and FLX exhibit both basic character, however ATE's log P value is lower than 1 (0.53±0.26),  
 210 what is typical for hydrophilic compounds, whereas FLX (4.16 ±0.26) is high lipophylic compound  
 211 (log P>3). For this reason, they have affinity for different types of sorbents. Three SPE cartridges,  
 212 including Polymeric RP (Strata-X), HLB (Oasis) and silica gel modified with NH<sub>2</sub> groups (Merck),  
 213 were investigated in this work. The experiment was conducted using 50 mL of ultrapure water,

214 INF and EFF, which were spiked at  $10 \text{ ng mL}^{-1}$  level of each analyte. The recoveries were  
 215 calculated according to following equation:  $\% \text{ Recovery} = ((C_{\text{spiked and extracted}} - C_{\text{non spiked}}) / (C_{\text{spiked before extraction}} - C_{\text{non spiked}})) * 100\%$ , where  $C_{\text{spiked and extracted}}$  is concentration of analytes in spiked samples  
 216 after extraction,  $C_{\text{spiked before extraction}}$  is concentration of analytes in spiked samples before extraction  
 217 and  $C_{\text{non spiked}}$  is concentration of analytes in non-spiked samples. The recovery calculated in that  
 218 way represents the loss arising from extraction step, excluding any losses by instrumental variations  
 219 (e.g. matrix effects in ionization chamber). The results of SPE recoveries are presented in Fig 1.  
 220 The results from NH2 cartridges were excluded due to the very low recoveries (5-36%).  
 221



222  
 223 *Fig 1. Effect on SPE sorbents on recovery of analytes in ultrapure water, influent and effluent samples spiked*  
 224 *with ATE and FLX at  $10 \text{ ng mL}^{-1}$ .*

225 The recoveries higher than 100% were obtained for S-ATE (Oasis HLB) and R-ATE  
 226 (Oasis HLB and Strata-X) extracted from ultrapure water, whereas the recoveries of S-FLX and  
 227 R-FLX were in the range of 50-70%. In case of real samples, lower recoveries (<50%) were  
 228 obtained where the SPE was performed using Oasis HLB SPE columns. For this reason, further  
 229 experiments were evaluated by using Strata-X SPE columns. Due to basic character of the analyzed  
 230 compounds, the experiments were conducted under the pH of 8.0. However, to reduce the labor  
 231 consumption of sample preparation step and the amount of solvents released to environment,  
 232 d-SPE procedure was developed. Three different sorbents, including Polymeric RP, silica gel

233 modified with C18 group and silica gel modified with NH<sub>2</sub> groups, were used in order to get the  
234 highest recoveries. Since ATE has higher affinity to hydrophilic sorbents than hydrophobic ones,  
235 it was decided to combined NH<sub>2</sub> sorbent with Polymer RP or C18 sorbent. The experiment was  
236 conducted using 45 mL of ultrapure water, INF and EFF, which were spiked at 10 ng mL<sup>-1</sup> level of  
237 each analyte and 200mg of sorbent. First d-SPE procedure was generally based on SPE procedure.  
238 The recoveries were calculated as previously. The lowest recoveries of ATE were obtained in case  
239 of mixture of C18 and NH<sub>2</sub> sorbents (1:1). Thus, this approach was excluded from further  
240 experiments. To develop more environmentally friendly (greener) sample preparation procedure,  
241 it was decided to reduce the amount of solvent used during desorption process. Hence, two  
242 difference volumes of extraction solvents applied to desorb the analytes from the sorbent were  
243 used: 3 mL (1,5 mL of methanol and 1,5 mL of 5% ammonia solution in methanol) and 6 mL  
244 (3 mL of methanol, 1,5 mL of mixture of acetone, methanol and ethyl acetate (2:2:1 v/v) and  
245 1,5 mL of 5% ammonia solution in methanol). Extracts were obtained from 45 mL of ultrapure  
246 water spiked at 10 ng mL<sup>-1</sup> level of each targeted analyte. The results are presented in Fig.2.

247 High recoveries were obtained for FLX desorbed from Polymeric RP-NH<sub>2</sub> sorbent,  
248 whereas in the case of ATE, the recoveries were lower than 30%. A significant increase in recovery  
249 values was observed for ATE desorbed from Polymeric RP sorbent with 6 mL of solvents. A slight  
250 decrease (up to 5%) was noticed for FLX desorbed from Polymeric RP sorbent with 6 mL of  
251 solvents in comparison with Polymeric RP-NH<sub>2</sub> mixture. Hence, Polymeric RP sorbent was chosen  
252 as a preferred one, and 6 mL of solvents were decided to use as a minimum required to receive  
253 satisfactory results.

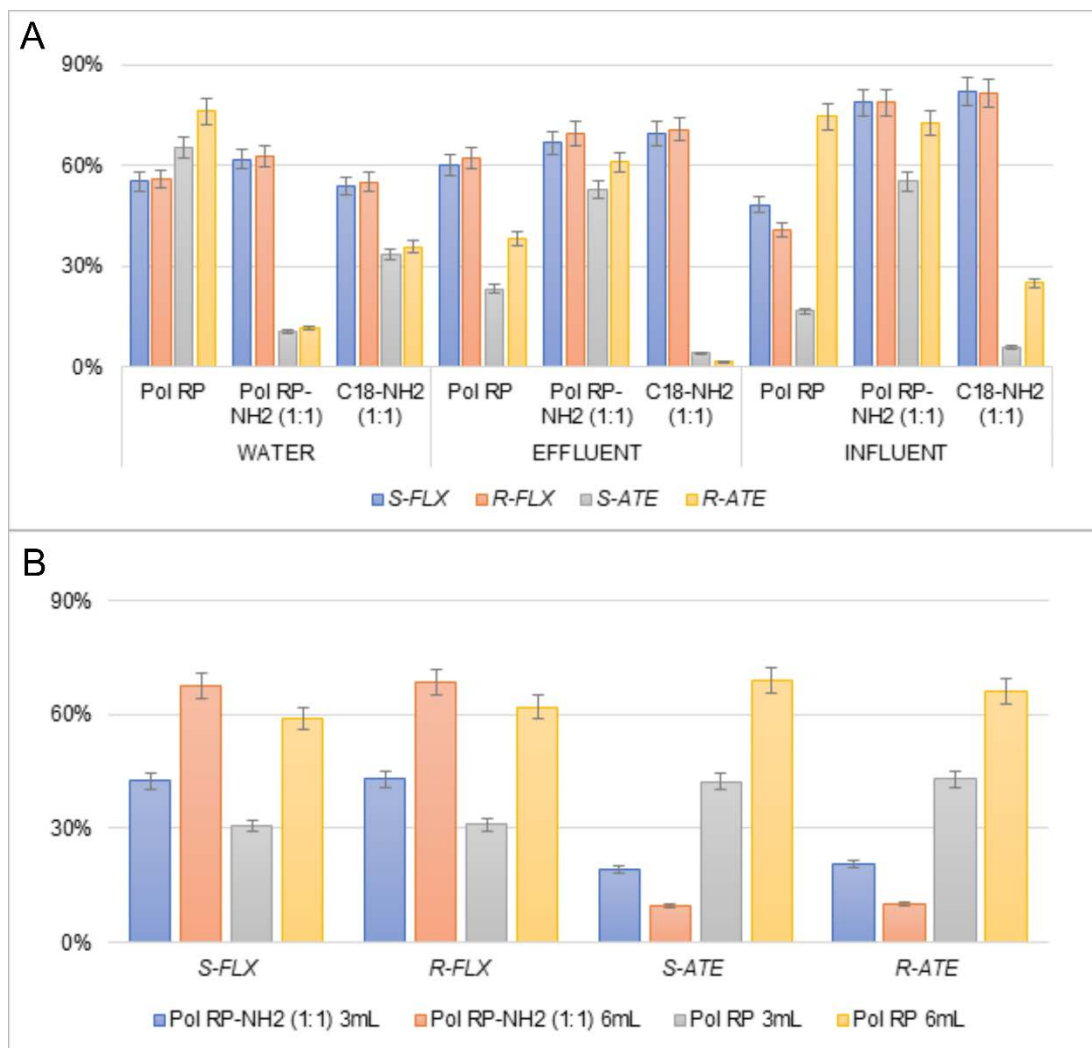


Fig 1. *d*-SPE optimization results presented as recoveries: a) selection of type of sorbent; b) optimization of extraction solvents volume.

### 3.4. Matrix effect on method performance

Due to the complexity of wastewater sample matrix, LC-MS response may be subjected to signal enhancement or suppression caused by the presence of interferents in the samples that affects analyte ionization. Therefore, the evaluation of matrix effects (%ME) is crucial to perform the reproducible and accurate quantitative analysis. In this study, %ME was evaluated by comparing the signal intensity of spiked sample extract ( $A_{spiked}$ ) with response of standard in water ( $A_{solvent}$ ) at the same concentration, according to following equation  $\%ME = ((A_{spiked} - A_{real\ sample}) / A_{solvent} - 1) * 100\%$ . The method was carried out with INF and EFF

265 sample by adding 10 ng mL<sup>-1</sup> concentration of FLX and ATE. The obtained calculation provided  
266 information whether there was ionization enhancement (%ME> 0%) or ionization suppression  
267 (%ME<0%).

268 As it was expected, %ME in INF where higher than those in EFF samples. High signal  
269 suppression (-79% and 81%) was observed for ATE and FLX added to raw wastewater, while the  
270 relatively small (10-24%) ion enhancement was showed in EFF. The highest signal enhancement  
271 was obtained for *S*-ATE (24%), what may be related to the characteristics of matrix components.

### 272 3.5. Quantitative Analysis of Atenolol and Fluoxetine

273 ATE and FLX are thought to be among the most frequently detected pharmaceuticals in  
274 environmental samples [17]. ATE is often used to treat high blood pressure (hypertension) and  
275 congestive heart failure, whereas FLX belongs to a group of selective serotonin reuptake inhibitors  
276 (SSRIs), used to treat depression. Both depression and hypertension are civilization diseases that  
277 affect millions of people around the world [18]. Due to this fact, they are often found in wastewater  
278 INF and EFF samples as well as in sludge samples [15,19–21]. During these studies, samples of  
279 INF and EFF were collected in different seasons in order to monitor the presence of ATE and FLX  
280 at enantiomeric level. The developed and validated chiral LC-MS method was used for the  
281 analysis and the positive confirmation of all enantiomers. The transition ratio between the  
282 precursor ion *m/z* and the second most abundant fragment was based on European Commission  
283 Decision 2002/657/EC. In the case of these studies, the elution order of enantiomers was obtained  
284 from the literature [1,15] as *S* and *R*, respectively. The obtained results are presented in Table 4.  
285 Due to chirality of studied compounds, enantiomeric fraction (EF) was evaluated using following  
286 equation:  $EF = (E_1 / (E_1 + E_2))$ , where  $E_1$  and  $E_2$  are the fractions of the first and second eluting  
287 enantiomer respectively. Considering the racemate, the EF value should be 0.5, whereas in case  
288 of enantiopure compound the value of EF is 1.0 or 0 [22]. The EF values are presented in  
289 Table 5.

290 All concentrations of determined ATE and FLX enantiomers were in the range of  
291 0.4-7.2 µg L<sup>-1</sup>. The highest concentration was for *R*-ATE detected in INF samples collected in the  
292 summer, whereas the lowest concentrations of all enantiomers (0.4-0.7 µg L<sup>-1</sup>) were found in EFF  
293 samples gathered in autumn. The concentration of *S*-FLX was generally higher than *R*-FLX in  
294 both treated and untreated wastewater. The same situation was observed in Sweden as well as in



295 the UK [15,19]. The *S*-FLX is considered to be more toxic to aquatic organism, therefore its  
296 presence in environmental waters raises concerns [19,20]. ATE was found to be season dependent,  
297 because the enrichment of *R*-ATE (EF= 0.3-0.4) was noticed in samples collected in spring and  
298 summer, whereas the slight increase in enrichment of *S*-ATE (EF= 0.53-0.63) was observed in  
299 samples collected in autumn and spring. ATE is sold as a drug in both racemate and *S*-enantiomer  
300 form, hence the reason of *R*-enrichment is presently unknown. Probably it is attributed to many  
301 factors, such as wastewater content or operational condition of WWTP. According to literature,  
302 ATE is mainly detected in INF and EFF samples as a racemate [19,20,23,24]. Due to this fact,  
303 further investigation should be performed to confirm the season dependence of ATE in wastewater  
304 in Poland.

305 The removal efficiency of ATE enantiomers ranges between 75 and 85% in all seasons,  
306 except from summer. The same situation can be observed for FLX enantiomers, where removal  
307 efficiency do not exceed 10%. In other cases, the slightly higher removal of *R*-FLX was observed.  
308 However, no significant changes in EF values of ATE and FLX after wastewater treatment were  
309 observed.

310 **Table 4.** Sample analysis results presented as concentration at [ $\mu\text{g L}^{-1}$ ] level

	Spring		Summer		Autumn		Winter	
	INF	EFF	INF	EFF	INF	EFF	INF	EFF
<i>S</i> -ATE	4.1084±0.0052	0.803±0.025	3.08±0.28	2.65±0.12	2.506±0.024	0.646±0.081	4.34±0.67	0.6517±0.0068
<i>R</i> -ATE	5.105±0.081	1.148±0.026	7.2±1.2	5.25±0.37	2.244±0.074	0.418±0.064	3.708±0.083	0.690±0.028
<i>S</i> -FLX	1.0181±0.0022	0.798±0.052	2.81±0.45	2.58±0.37	0.97±0.13	0.704±0.044	1.55±0.25	0.8880±0.0097
<i>R</i> -FLX	1.55±0.72	0.568±0.032	1.46±0.17	1.43±0.31	0.64±0.13	0.485±0.044	1.55±0.72	0.534±0.012

311 **Table 5.** Enantiomeric factors of ATE and FLX calculated for different seasons

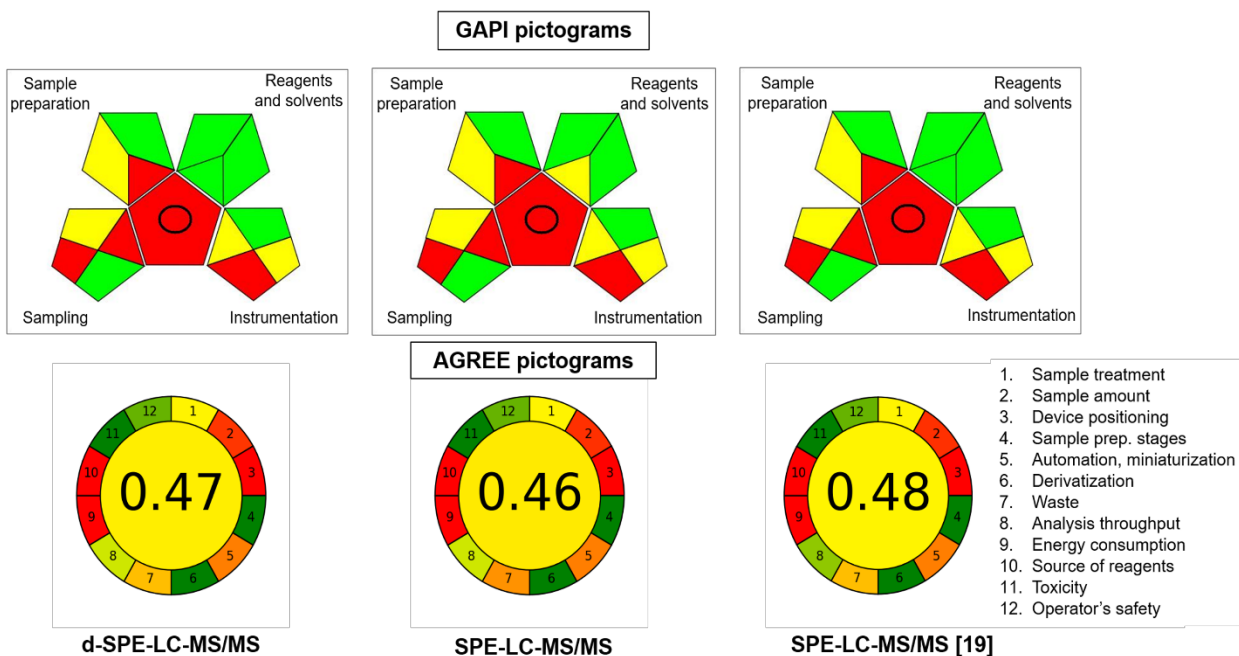
	Spring		Summer		Autumn		Winter	
	INF	EFF	INF	EFF	INF	EFF	INF	EFF
ATE	0.45	0.41	0.30	0.34	0.53	0.61	0.54	0.49
FLX	0.40	0.48	0.66	0.64	0.60	0.59	0.50	0.62

312



### 313 3.5. Assessment of greenness of developed methods

314 To evaluate a 'green' character of developed methods, GAPI and Analytical GREENness  
315 (AGREE) calculator were applied. The GAPI is an index to "green assessment" of analytical  
316 protocol in terms of the amount and type of waste, environmental hazard, chemical health as well  
317 as energy requirements. The results of this assessment are presented in pictorial form covering all  
318 stages of the methodology, from sampling to final determination [13,25]. The second tool,  
319 Analytical GREENness calculator, is a new assessment approach proposed by Pena-Pereira et al.  
320 [26]. The evaluation criteria of AGREE were taken from the twelve principles of green analytical  
321 chemistry and transformed into 0-1 range. The higher average score the method receives, the  
322 greener it is. In these studies, SPE-LC-MS/MS method was compared with d-SPE- LC-MS/MS  
323 method to assess the effect of sample preparation change on greenness of method. In addition, the  
324 greener procedure was compared to one reported previously in literature [19]. The results from  
325 GAPI and AGREE tools are presented in Fig 3.



326  
327 Fig. 3. Greenness assessment of developed methods for chiral separation using GAPI and AGREE tools

328 LC-MS is generally not environmentally friendly technique due to a large amount of  
329 solvent used and high energy consumption. Nevertheless, the use of GAPI tool allowed to compare

330 different methods and select the greener approach for this research. In the  
331 case of d-SPE-LC-MS/MS method, there is a significant difference in the 'Reagent and Solvents'  
332 part. Meaning, to perform d-SPE extraction the amount of solvents required is much smaller.  
333 According to the scores obtained in AGREE tool (both around 0.50), there is no significant  
334 difference between these two approaches, however, the final score of d-SPE-LC-MS/MS method  
335 (score= 0.47) is slightly higher than SPE-LC-MS/MS method (score= 0.46). Both methods have  
336 the same strong drawbacks: off-line sampling, high energy consumption and use of reagents from  
337 non-green sources. On the other hand, the use of a vancomycin packed column for the chiral  
338 separation allows to avoid the derivatization step, thus prevents the release of hazardous substances  
339 into the environment. Moreover, water and methanol, that are considered as green solvents, were  
340 used both in sample preparation and analysis steps. Still, two parameters differ these methods: the  
341 amount of sample required and amount of waste generated which is smaller in case of d-SPE.  
342 Hence, it was concluded that the d-SPE-LC-MS/MS method is marginally greener than the method  
343 with SPE extraction in a sample preparation step.

344 In comparison with the method developed by Evans et al. [19], there is no significant  
345 difference between its GAPI pictogram and d-SPE approach pictogram. According to AGREE  
346 results, the method reported earlier obtained a better score. Despite similar advantages and  
347 disadvantages in terms of greenness, the method from literature has higher analysis throughput,  
348 which slightly influence the final result. However, the method reported in this paper is newly  
349 developed, therefore extensive research should be carried out in order to broaden the range of  
350 determining analytes as a part of future studies.

### 351 **3.6. Environmental Application of RP-LC-MS/MS Method**

352 The first method developed in this studies was applied to determine 6 pharmaceuticals in  
353 INF and EFF samples collected in different seasons. All selected compounds were found in INF  
354 samples, whereas only KET was not detected in EFF samples, expect from those gathered in winter.  
355 Detection of profens (IBU, KET) in winter EFF is connected with flu and cold season. Due to the  
356 fact that KET and IBU belong do NSAIDs group, they are generally easily available and often  
357 taken to reduce the fever. No significant difference was observed in the occurrence of ATE,  
358 FLX and OME in both INF and EFF samples collected in various seasons. This is probably related



359 to long-term treatment with these compounds and explains the constant release of them into the  
360 environment. The last analyte, OFL, was found in every INF and EFF sample. OFL is useful  
361 antibiotic for the treatment of a numerous of bacterial infections, so its presence in wastewater is  
362 often confirmed [27].

#### 363 **4. Summary**

364 The presented studies show the occurrence of six frequently prescribed pharmaceuticals  
365 in wastewater samples and chiral separation of ATE and FLX. Both analytes were monitored  
366 seasonally at the enantiomeric level. The enantiomeric compositions of analysed compounds  
367 presented racemic to weakly enantioselective, with the highest EF value (0.66) for FLX detected  
368 in the summer. It was also noticed that the content of ATE enantiomers in wastewater may be  
369 seasonal dependent, however, further investigations to confirm it are still required. In order to  
370 reduce the solvent consumption and time-consuming of sample preparation step, d-SPE protocol  
371 was developed. Due to the trend of working in accordance with the idea of a sustainable  
372 environment, the evaluation of environmental impact of these methods was performed.  
373 The assessment of greenness of proposed methods was carried out using two different tools: GAPI  
374 and AGREE. In both cases, the results indicates that using d-SPE instead of SPE has a slightly  
375 lower impact on the environment. Moreover, both final scores of AGREE were relatively high  
376 (around 0.50), which can be interpreted as quite good results as the categories of this tool are very  
377 strict and demanding. However, further research to develop a faster, cheaper and more  
378 environmentally friendly procedure for chiral separation should be performed.

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386 **7. Literature**

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478

Supplementary material

**Analysis of chiral pharmaceutical residues in influent and effluent samples at racemic and enantiomeric level using liquid chromatography-tandem mass spectrometry**

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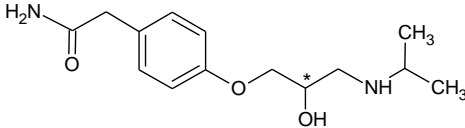
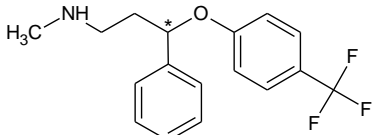
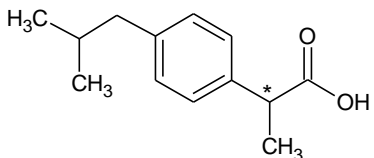
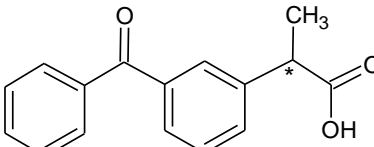
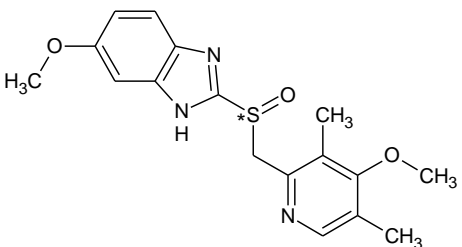
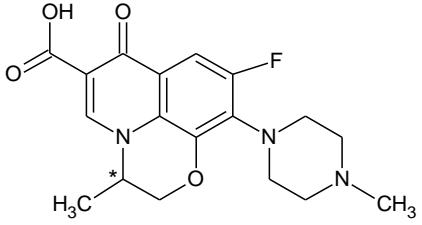
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**Table S1**

Analytes selected for analysis.

Compound	Indication	Molecular weight (g/mol)	pKa	Structure
Atenolol (ATE)	$\beta$ -blocker	266	9.6	
Fluoxetine (FLX)	serotonin reuptake inhibitor	309.3	9.8	
Ibuprofen (IBU)	NSAID	206.28	5.3	
Ketoprofen (KET)	NSAID	254.28	4.45	
Omeprazole (OME)	proton pump inhibitor	345.4	4.77-9.29	
Ofloxacin (OFL)	antibiotic	361.4	5.97-9.28	

\* represents chiral centre

**Table S2**

Lipophilicity descriptors calculated using the ALOGPs 2.1 program

Descriptor	A LOGPs	ACLOG P	A LOG P	M LOGP	XLOG P2	XLOG P3	Log P Av.	Log S
Analyte								
ATE	0.57	0.41	0.67	0.93	0.46	0.16	0.53+0. 26	-2.41
FLX	4.09	3.96	4.03	4.15	4.65	4.05	4.16+0. 25	-4.41