

1 **Multiclass determination of drug residues in water and fish for bioaccumulation potential assessment**

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25 **Abstract**

26 In this work, a wide-scope liquid chromatography-tandem mass spectrometry (LC-MS/MS)
27 method for the quantitative determination of environmental levels of multiclass drugs and their
28 metabolites in water and fish samples was developed. The method allowed the reliable
29 determination of 44 drugs, covering a rather wide range of chemistries and physicochemical
30 characteristics. In order to obtain a reliable and robust analytical protocol, different combinations
31 of extraction and cleanup techniques were systematically examined. Aqueous samples were
32 extracted using a simple Oasis HLB SPE enrichment protocol with pH-optimized sample
33 percolation (pH 3). The extraction of cryo-homogenized biota samples was performed using
34 double extraction with MeOH basified with 0.5% NH₃, which allowed high extraction recoveries
35 for all target analytes. The problem of the coextracted lipid matrix, which is known to be the key
36 obstacle for reliable biota analysis, was systematically examined in a series of model cleanup
37 experiments. A combination of cryo-precipitation, filtration, and HLB SPE cleanup was proposed
38 as a protocol, which allowed reliable and robust analysis of all target compounds at low ng/g levels.
39 At the final conditions, the method which was validated at three concentration levels showed high
40 extraction recoveries (68 – 97%), acceptable matrix effects (12 – -32%), accuracies (81 – 129%),
41 and reproducibilities (3 – 32%) for all analytes. The developed method was used to determine drug
42 concentrations in river water and in feral freshwater fish, including whole fish and muscle tissue,
43 from the Sava River (Croatia), in order to estimate their corresponding bioaccumulation potential.
44 With respect to bioaccumulation potential in whole fish and fish muscle, the most relevant drugs
45 were lisinopril, sertraline, terbinafine, torsemide, diazepam, desloratadine, and loratadine with
46 estimated bioaccumulation factors ranging from 20 to 838 and from 1 to 431, respectively.

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48 Key words: pharmaceuticals; biota; surface water; organic contaminants; bioaccumulation

49 **1. Introduction**

50 The problem of the occurrence and fate of pharmaceutically active compounds (PhACs) in the
51 aquatic environment has been recognized as an emerging concern for over three decades [1,2]. The
52 overall rapid increase in drug production and consumption [3,4] and incomplete removal of drugs
53 during conventional sewage treatment [5,6] warrants a continuously increasing input of PhACs
54 into the aquatic environment and result in their widespread occurrence and pseudo-persistence in
55 different environmental compartments [7–9]. Moreover, the number of PhACs with wide
56 therapeutic use is rapidly increasing and includes several thousand regulated substances. There is
57 accumulating evidence in the literature that drugs belong to the most prominent categories of
58 emerging environmental contaminants. However, the data on drug bioaccumulation in aquatic
59 organisms are still comparatively scarce. In recent years, the emphasis has been directed at the
60 interactions of drug residues with aquatic organisms, including assessment of the adverse effects
61 and bioaccumulation [10]. As suggested by Miller et al. [11], the possible toxic effects should be
62 determined by the internal concentration of PhACs in the organism, rather than the exposure
63 concentrations in water. Several reports have shown that some drugs as well as their metabolites
64 and transformation products can accumulate in the tissues of aquatic organisms such as fish,
65 crustaceans, and mollusks [12–16]. Moreover, the studies indicated that chronic exposure of fish
66 and other organisms to low concentrations of PhACs can lead to alteration of important
67 physiological processes, such as development, reproduction, and nervous system functions [17–
68 20]. Consequently, the methods which allow highly specific analysis of PhACs in biological
69 tissues are in high demand.

70 In the last few years, a number of analytical methods have been developed for the determination
71 of PhACs in biotic matrices. A review by Álvarez-Ruiz and Picó [21] compiled the achievements
72 in the sample preparation and instrumental methods used for the analysis of emerging and related
73 pollutants in the aquatic biota and highlighted some key points to be addressed in future research,
74 such as improvement of the fat removal to reduce matrix effects and development of multiresidue
75 methods that allow simultaneous determination of different classes of PhACs. Recent literature
76 shows that, although the instrumental analysis of complex mixtures of pharmaceuticals and
77 personal care products using coupled chromatography-mass spectrometry techniques has become
78 a widely available and routine tool, the sample preparation of complex matrices such as biological
79 tissues, including extraction and extract cleanup, remains a challenge [21]. The biota analysis has

80 two difficult and frequently conflicting aspects: the practical requirements for the high sample
81 throughput and ease of use, as well as the requirement for efficient and selective removal of matrix
82 effects, which frequently involves laborious procedures with multiple cleanup steps. Some recently
83 published methods put the emphasis on an extensive removal of matrix components as a key
84 prerequisite [22–25], often resulting in lengthy and tedious procedures. Therefore, some authors
85 proposed alternative procedures which favored the simplicity and suitability for high throughput
86 routine analysis [26,27], often at the expense of some analytical quality indicators (highly variable
87 absolute recoveries, lower reproducibility) and reduced versatility (suboptimal recoveries of some
88 problematic analytes).

89 Consequently, the main objectives of the present study included: i) the development and validation
90 of the analytical methods for multiclass analysis of 44 drug residues (Table S1) in water and fish
91 tissue, with a special emphasis on the sample cleanup for biological matrices in order to provide a
92 tool for the assessment of the bioaccumulation potential, and ii) application of the developed
93 method for the analyses of field samples, including both river water and feral fish samples collected
94 from the Sava River.

95 The selection of the target drugs was based on the recent report of the Croatian regulation agency
96 HALMED [3], considering the consumption of the most prominent drugs across several
97 therapeutic drug categories. In addition, several commonly abused drugs, such as 3,4-
98 methylenedioxy-methamphetamine (MDMA), and cocaine, as well as some frequently abused
99 opioids were also included.

100

101 **2. Materials and methods**

102 2.1. Chemicals and materials

103 HPLC grade methanol (MeOH), HPLC grade acetonitrile (ACN), and LC-MS grade water were
104 purchased from VWR International. LC-MS grade formic acid, ACS grade formic acids,
105 ammonium formate ($\geq 99.9\%$), and 7 N ammonia solution in MeOH were purchased from Sigma-
106 Aldrich. A 25% aqueous ammonia solution (p.a.) was supplied by Merck. Certified standard
107 solutions were applied for most of the target analytes (1 g/L) and their isotopically labeled
108 analogues (IS; 0.1 g/L) during the method development and sample analyses (for details regarding
109 suppliers see Table 1). In case such certified reference materials were not available, analytical
110 standards were purchased as solids of high purity ($>95\%$) and their individual stock solutions (1

111 g/L) were prepared in the appropriate solvent. Two working multianalyte solutions containing 44
112 target analytes and 35 IS were prepared in MeOH at concentrations of 10 mg/L and 5 mg/L,
113 respectively. All solutions were kept in the dark at -20 °C. The Oasis HLB solid-phase extraction
114 cartridges (200 mg/6 mL) and PTFE Acrodisc Mini Syringe Filters (0.2 µm/13 mm and 0.45 µm/13
115 mm) were purchased from Waters, while glass-fiber filters (GF/F and GF/D) were obtained from
116 Whatman. Several SPE materials, applied during a method development (amino silica, PSA, and
117 C₁₈) were supplied by Phenomenex (Torrance, USA).

118

119 2.2. Method development

120 2.2.1. Extraction of water samples

121 Development of the method for the analysis of surface water samples was performed using spiked
122 water samples collected from a pristine mountain creek. Pre-preparation of water samples included
123 homogenization and filtration through glass-fiber filters (GF/D) to remove the suspended matter.
124 Solid-phase extraction (SPE) was performed using Oasis HLB cartridges. The optimization of the
125 SPE procedure was performed using model freshwater samples (500 mL), which were prepared
126 by spiking the known amounts of target analytes and IS (50 ng/L) to the homogenized creek water
127 before filtration. The model experiments included selection of optimal pH conditions (pH 3 and
128 pH 7) and cartridge elution conditions (selection of solvent type and volume). The final validation
129 was performed at three concentration levels (5 ng/L, 50 ng/L, and 500 ng/L), covering a broad
130 concentration range.

131 In the final procedure (Fig. 1a), pH of the sample was adjusted to 3 using formic acid, the sample
132 was percolated (≤ 5 mL/min) through prewashed and preconditioned Oasis HLB cartridges. The
133 cartridges were prewashed with 6 mL of 1% ammonia solution in MeOH, followed by 6 mL of
134 MeOH, and preconditioned with 6 mL of ultrapure water, and 6 mL of acidified water (pH 3).
135 After the sample percolation, cartridges were washed with 6 mL of acidified water and dried for
136 30 min under a stream of N₂. Adsorbed analytes and surrogates were subsequently eluted using 2
137 mL of MeOH and 4 mL of 0.2% ammonia in MeOH. The eluates were combined and evaporated
138 to dryness at 40 °C under the gentle stream of N₂ and a dry residue was reconstituted in 0.5 mL of
139 the water/MeOH mixture (60:40, v/v) containing 50 mM ammonium formate and 0.2% of formic
140 acid. Before LC/MS analysis, final extracts were filtered through PTFE Acrodisc Mini Syringe
141 Filters.

142

143 2.2.2. Extraction of fish samples

144 The development of the method for the analysis of fish tissue was performed using cryo-milled
145 (SPEX SamplePrep 6875 Freezer/Mill, USA) pooled fish muscle of the European chub (*Squalius*
146 *cephalus*) that was obtained by electrofishing from the non-polluted Plitvice Lakes. Versatile
147 extraction conditions, including different types and combinations of extraction solvents, number
148 of the extraction cycles as well as sample type (dry and wet muscle samples) were tested before
149 the selection of final extraction conditions. The two key parameters considered in the process of
150 method optimization were extraction and cleanup recoveries and the impact of the matrix effect.
151 However, practical aspects affecting sample throughput were also considered. The model samples
152 for the method development were prepared by spiking homogenized fish muscle samples with the
153 MeOH solution of target analytes (addition of 5 μ L). The spiked samples were left for 30 min to
154 equilibrate and evaporate MeOH before being subjected to ultrasound-assisted extraction (UAE;
155 Branson 3800, USA).

156 The final extraction protocol included UAE of 1 g of wet cryo-milled fish tissue (fish muscle and
157 whole fish) with 0.5% ammonia in MeOH (2 x 5 mL) in 2 consecutive extraction cycles of 15 min
158 (Fig. 1b). After each cycle, samples were centrifuged for 15 min at 3392 ref. Supernatants were
159 collected, pooled, evaporated to approximately 1 mL, and left overnight in the freezer (-20 °C).

160 The validation of the final procedure was performed by analyzing model fish samples spiked at
161 three concentration levels (10 ng/g, 50 ng/g, and 250 ng/g) in quadruplicate.

162

163 2.2.3. Cleanup of fish sample extracts

164 In order to reduce the coextracted interfering matrix components, several cleanup approaches were
165 tested: 1) Liquid-liquid extraction (LLE) of the final extract with n-hexane as proposed by Boulard
166 et al. [25] (0.5 mL of n-hexane added to 1 mL of final extract); 2) silica gel fractionation of the
167 extract using a modified method by Terzic and Ahel [28] (sequential elution with n-hexane,
168 dichloromethane, 0.5% ammonia in MeOH, and 1% formic acid in MeOH); 3) dispersive SPE of
169 UAE extract (1 mL) with aminosilica, C₁₈, and PSA sorbents as in the QuEChERS approach[24];
170 4) cryo-precipitation of preconcentrated UAE extract overnight, and 5) catch-and-release SPE
171 (Oasis HLB) of aqueous solution of preconcentrated UAE extract (100:1) based on the procedure
172 developed for water samples, including filtration on GF/F before SPE.

173 The final cleanup procedure included a combination of three cleanup steps (Fig. 1b): 1) overnight
174 cryo-precipitation (at -20 °C) from the concentrated UAE extracts (1 mL) followed by
175 centrifugation at 21382 rcf at 0 °C (Hettich, Germany), 2) dilution of the supernatant with 100 mL
176 of acidified water (pH 3) followed by filtration (GF/F), and 3) subsequent SPE using Oasis HLB
177 cartridges. The residue was reconstituted in the final injection solvent (1 mL of 50 mM ammonia
178 formate and 0.2% of formic acid in water and MeOH, 60:40 (v/v)) and filtered through PTFE (0.2
179 µm) filters before LC/MS analysis.

180

181 2.3. Liquid chromatography and mass spectrometry analysis (LC/MS)

182 Chromatographic separations were carried out using a Thermo Finnigan Surveyor LC system,
183 equipped with a Phenomenex Synergi Polar-RP column (150 mm × 3 mm, 4 µm, 80 Å). Different
184 eluents and gradient programs were tested, in order to optimize the separation of the target
185 analytes. In the final protocol, the target compounds were separated using water (A) and MeOH
186 (B) as elution solvents, both containing 0.1% (v/v) of formic acid, at a flow rate of 0.4 mL/min.
187 Gradient conditions were as follows: 0 – 8 min from 10% to 40% of eluent B, 8 – 28 min from
188 40% to 60% of B, 28 – 38 min from 60% to 80% of B, 38 – 40 min from 80% to 90% of B, 3 min
189 of isocratic hold at 90% of B, 43 – 44 min from 90% to 10% of B, and 44 – 54 min 10% of B. The
190 injection volume was 15 µL. The detection of target compounds was performed using a TSQ AM
191 triple quadrupole mass spectrometer (Thermo Electron, USA) equipped with an ESI interface. All
192 analyses were carried out in positive ionization mode. The MS parameters were as follows: spray
193 voltage, 3500 V; capillary temperature, 330 °C; sheath gas (N₂), 40; auxiliary gas (N₂), 10. Ar was
194 used as a collision gas. The acquisition was performed in multiple reaction mode (MRM). Two
195 selected precursor/product ion transitions were monitored for each compound (Table 1) and
196 subsequently applied for quantification and confirmation purposes, respectively. Each analytical
197 batch included quality control samples as well as instrumental and procedural blanks.

198

199

200 2.4. Quantification and method validation

201 Quantification of target compounds was performed using internal standard calibration. The method
202 included 35 isotopically labeled surrogates (IS). For 9 analytes, whose IS were not available, the
203 most appropriate IS were chosen among the available, based on their structural, physicochemical,

204 and/or chromatographic properties, considering their extraction recoveries and susceptibility to
205 matrix effects (Table 1).

206 Linearity, instrumental detection limit (IDL), method quantification limit (MQL), extraction
207 recovery, accuracy, repeatability (precision), and matrix effect were systematically evaluated for
208 each individual analyte as the key criteria during the method validation. The linearity of the
209 response was determined by injecting standard solutions containing fixed concentrations of
210 internal standards (50 ng/mL) and analytes in concentrations ranging from 0.1 to 5 000 ng/mL
211 using fifteen-point internal standard calibration curves. Repeated injections of the standard
212 solutions at concentrations close to the detection limit until the signal-to-noise (S/N) ratio was
213 approximately 3 were used to assess IDLs. MQL was defined as the analyte concentration in the
214 sample which provided 8-fold or higher signal values as compared to the noise, determined in the
215 matrix-matched standard solution and considering extraction recovery. Extraction recoveries were
216 determined by comparing the analyte responses of the target compounds into the corresponding
217 samples (surface water or fish muscle) as follows:

$$218 \text{ Extraction recovery (\%)} = \frac{A_{be} - A_{orig}}{A_{ae} - A_{orig}} \times 100\%$$

219 where (A_{ae}) is the average response of the analyte spiked to the extract after extraction and (A_{be})
220 is the response of the analyte spiked before the extraction. Both responses were corrected for the
221 average response of the analyte in the original sample (A_{orig}), if any.

222 The method accuracy was calculated using the following equation:

$$223 \text{ Accuracy (\%)} = \frac{c_2 - c_1}{c_0} \times 100\%$$

224 where c_0 , c_1 , and c_2 represent the nominal spiked concentration, average concentration measured
225 in the original sample, and average concentration measured in the spiked sample, respectively. The
226 repeatability was calculated as the relative standard deviation (RSD) of the sample analyses
227 performed in quadruplicates.

228 The matrix effect was evaluated as the ratio of the average response of the target compound spiked
229 into the final extracts (A_{fe}) and the average response in a matrix-free standard solution having the
230 same concentration (A_{std}) as indicated in the following equation:

$$231 \text{ Matrix effect (\%)} = \frac{A_{fe} - A_{orig} - A_{std}}{A_{std}} \times 100\%$$

232

233 2.5. Sample collection and storage

234 The developed analytical method was applied to assess the bioaccumulation of the target
235 pharmaceutical compounds in the feral fish from the Sava River, Croatia. The samples were
236 collected in the vicinity of the wastewater outlet of the central wastewater treatment plant (WWTP)
237 of the city of Zagreb (location Hruscica). The sampling was performed in the autumn of 2021
238 (11/11/21) and in the spring of 2022 (18/05/22). During each sampling day, three grab water
239 samples were collected (at the beginning, in the middle, and at the end of the electrofishing). The
240 collected water samples were kept in the refrigerator (4 °C) and were processed within 24 h after
241 collection.

242 Electrofishing (Hans Grassl, EL63 II GI) was applied to collect fish samples at the same location.
243 It was performed in accordance with Croatian regulation (Standard HRN EN 14011:2003).
244 Stunned fish were examined and dissected in the field immediately after sampling [29]. Before the
245 dissection, the basic biometric data were recorded, including body mass and the total length of the
246 fish. Information about species and morphological measurements of caught fish are presented in
247 the Supplementary Material (Table S2).

248 Collected fish tissue samples were placed in labeled HD polypropylene plastic vessels or bags and
249 stored in the refrigerator. Upon return to the laboratory, the samples were kept at -20 °C until
250 homogenized and pulverized using Freezer/Mill cryogenic grinder in order to facilitate a more
251 efficient extraction.

252

253 **3. Results and discussion**

254 3.1. Optimization of SPE for the analysis of water samples

255 In this study, the Oasis HLB cartridges, which are well-known for their versatility in the field of
256 multiresidue analysis of drugs in environmental samples [30–32], were applied for the enrichment
257 of the target compounds from aqueous samples as well as for the cleanup of fish extracts. This
258 allowed an efficient (>50%) simultaneous extraction for most of the target compounds,
259 characterized by a wide range of physicochemical properties with log K_{ow} values ranging from -
260 1.2 to 6.4 and pK_a values from 0.7 to 14 (Table S1). In order to optimize the extraction conditions,
261 the extraction efficiency was evaluated at pH 3 and pH 7 and the results are presented in Table S3.
262 The extraction recoveries for 6 out of the 44 analytes (lisinopril, norfloxacin, ciprofloxacin, EDDP,
263 amlodipine, and atorvastatin) were less than 50% at pH 7. The recoveries of these analytes at acidic
264 conditions (pH 3) were significantly improved but this was accompanied by rather poor extraction

265 efficiencies for the most polar analytes, cotinine (13%) and morphine (5%), from larger volume
266 water samples (500 mL). Assuming that these two polar analytes are comparatively less important
267 regarding their bioaccumulation potential, and considering better extraction yields and analytical
268 reproducibility (average RSD = 7%) for other target compounds, the acidic SPE was chosen as a
269 standard extraction procedure in this study.

270 In preliminary experiments, the elution efficiency of the analytes adsorbed on the HLB sorbent
271 was optimized using different solvent combinations (Table S3). Most of the enriched analytes were
272 efficiently desorbed from the cartridge either with neutral or basified MeOH, however, some pH-
273 sensitive analytes preferred simple MeOH elution (e.g. EDDP), and some others (desloratadine,
274 norfloxacin, and ciprofloxacin) required basified MeOH for an efficient elution. The attempt to
275 enhance recoveries of fluoroquinolone antibiotics or atorvastatin by using a higher percentage of
276 ammonium (1%) in MeOH or by introducing a third elution step with acidic MeOH (1% HCOOH
277 in MeOH) did not show any significant improvements. Therefore, a sequential elution with 2 mL
278 of MeOH followed by 4 mL basified MeOH (0.2% NH₃ in MeOH) was selected for all further
279 analyses.

280

281 3.2. Optimization of extraction and cleanup procedures for the analysis of fish samples

282 Regarding the extraction step, most of the protocols developed for multiresidue trace determination
283 of pharmaceuticals in fish are based on ACN or MeOH as extraction solvents [11,21], with a
284 predominant use of neutral [22,33,34] and acidified solvents [26,27,35]. However, to the best of
285 our knowledge, basified solvents have not yet been systematically evaluated and applied for this
286 purpose in the literature. Based on our previous experience with solid environmental matrices,
287 such as sediments and biosolids, which showed enhanced extraction efficiencies for basic
288 compounds (e.g. macrolides and opioids), using basified organic solvents [36], this approach has
289 been applied in this work. The extraction experiments were performed by UAE in two consecutive
290 cycles since the results of the preliminary extraction experiments revealed significantly better
291 recoveries of certain analytes, including sertraline, terbinafine, valsartan, diazepam, loratadine,
292 atorvastatin when using two extraction cycles as compared to one extraction cycle. A summary of
293 the results achieved using double UAE with different extraction solvents is given in Fig. 2, while
294 the detailed data for all individual target analytes are given in Table S4. Generally, the results show
295 that the extraction recoveries in this study achieved by MeOH-based solvents were much higher

296 as compared to ACN-based solvents (Fig. 2; Table S4). This result is consistent with findings of
297 Huerta et al. [22], who indicated that MeOH had a higher extraction efficiency than ACN but
298 suggested that ultrasound-assisted extraction was not as efficient as pressurized liquid extraction
299 (PLE). However, a more recent report by Peña-Herrera et al. [24] showed that UAE provided good
300 recoveries for most of the target pharmaceuticals studied and concluded that there was no
301 significant difference between ACN and MeOH as extraction solvents. Nevertheless, a detailed
302 analysis of the extraction efficiencies with ACN-based solvents reported in that paper showed
303 unsatisfactory recoveries for some problematic compounds such as amlodipine, fenofibrate,
304 paroxetine, cephalexin, loratadine, and sertraline, which were omitted from their final method.
305 Some authors (e.g. Grabicová et al. [26]) suggested that addition of some modifying solvents, such
306 as ethanol and isopropanol, to ACN increased the extraction efficiencies of biota samples. To test
307 this hypothesis in basic conditions, two combinations of ammonia-basified ACN with acetone
308 (Fig. 2) or isopropanol (results not shown) were also examined. However, none of these solvent
309 combinations provided an improvement over the MeOH-based solvents for our set of target
310 analytes. In fact, the preferred and widespread use of ACN as an extraction solvent for
311 pharmaceuticals in the literature does not rely on its extraction potential for polar compounds but
312 on the selectivity vs lipid interferences. Consequently, MeOH-based solvents, including 0.5%
313 ammonia in MeOH, 0.5% ammonia in MeOH/acetone 3:1 (v/v), and 0.2% formic acid in MeOH
314 were selected for further method development. Fig. 2 shows that significantly better extraction
315 recoveries were obtained at basic extraction conditions as compared to the acidic ones, which
316 prevail in the literature [21], confirming the method improvement achieved in this work.
317 However, it should be stressed that MeOH-based solvents coextracted a much higher proportion
318 of interfering matrix substances, which resulted in a strong signal suppression (>50%) for a number
319 of analytes (Fig. 2; Table S5), especially when using MeOH/acetone mixture. This can
320 compromise the detection limits and reproducibility of measurements, which is in agreement with
321 other reports in the literature (e.g. Peña-Herrera et al. [24]). Therefore, 0.5% ammonia in MeOH
322 was selected as optimal, despite the slightly higher average recovery achieved by 0.5% ammonia
323 in MeOH/acetone 3:1 (v/v).

324 The selected extraction procedure was verified for both, dry and wet tissue samples, and the
325 extraction recoveries for individual compounds can be found in Table S6. For most analytes, high
326 recoveries were obtained for both sample types (53% to 91% and 68% to 97%, respectively) and

327 these were similar to or better than those reported in the recent literature [24–26]. Nevertheless,
328 the extraction recoveries of some analytes, including norfloxacin, ciprofloxacin, sertraline,
329 terbinafine, diazepam, and loratadine were significantly lower from the dry samples as compared
330 to the wet samples (Table S6). Therefore, the extraction of wet samples was selected for all further
331 analyses, including real samples from the Sava River.

332 At the selected extraction conditions (UAE, 2 extraction cycles with 0.5% ammonia in MeOH),
333 coextracted matrix substances in raw extracts caused a rather high signal suppression, which was
334 above 40% for 27 of 44 target analytes (Table S7). Consequently, several cleanup approaches were
335 tested in preliminary screening experiments as described in the experimental section and the results
336 are presented in Table S7. Among the tested approaches, the noteworthy results in terms of matrix
337 effect reduction were obtained only by silica gel fractionation and catch-and-release SPE with
338 Oasis HLB. However, despite the promising initial results, silica gel fractionation was rather prone
339 to clogging and appeared to be too me-consuming (2 h/fractionation) for a high throughput method.
340 Therefore, the standard SPE Oasis HLB protocol (pH 3), which was previously developed for
341 water sample analyses, was finally selected for further method optimization. It was shown that this
342 procedure significantly decreased the suppression for most of the analytes. However, it remained
343 relatively high (>40%) for 13 analytes, especially for desloratadine, sertraline, terbinafine, and
344 loratadine (>70%).

345 Assuming that the main components of the interfering tissue matrix were lipids, we systematically
346 examined two additional procedures with the potential to selectively remove lipids, without
347 unwanted losses of the target compounds. The first approach, which is based on the lower
348 solubility of lipids in organic solvents at low temperatures (cryo-precipitation), has been widely
349 applied in the analysis of trace contaminants, including pharmaceuticals [26,33]. The second
350 approach relies on the difference in their solubilities in the aqueous media, which is comparatively
351 higher for pharmaceuticals. The results achieved by these two procedures are presented in Table
352 S8 and Table S9.

353 The effects of the cryo-precipitation approach were examined using the concentrated UAE extracts
354 (1 mL MeOH) which were kept overnight at different temperatures (4 °C, -20 °C, and -80 °C) to
355 enhance the precipitation of lipids, followed by centrifugation at 3392 or 21382 rcf. The cryo-
356 precipitation was relatively inefficient in reducing signal suppression for most of the analytes, even
357 for the most rigorous procedure (-80 °C and 21 382 rpm), with 11 analytes showing suppression

358 higher than 40% (Table S8). Obviously, the cryo-precipitation from MeOH media is less efficient
359 than the precipitation from ACN extracts described in the literature [26].

360 The second approach using HLB SPE comprises the transfer of the extract into an aqueous
361 medium, which leads to the flocculation of the present lipids. The flocculation in our extracts
362 caused intensive clogging of the SPE cartridges, which extremely slowed down the SPE sample
363 cleanup step. In order to prevent clogging, SPE cleanup procedure was preceded by the filtration
364 of the aqueous extract solutions through GF/F filters. The preliminary model samples showed that
365 this procedure did not cause any significant losses of the target analytes. It turned out, that the
366 filtration step before SPE did not only resolve the cartridge clogging issue but also resulted in an
367 additional reduction of the signal suppression which dropped below 25% for 38 out of 44 analytes
368 and, even more important, below 40% for all target analytes. (Fig. 3; Table S9). The observed
369 improvement in the reduction of matrix effects can most probably be ascribed to the physical
370 removal of coextracted lipid compounds. A similar result was obtained by combining cryo-
371 precipitation and Oasis HLB SPE without additional filtration (Table S9).

372 However, in both cases, the suppression of the critical analytes was considered still relatively high,
373 which might pose a problem with lipid-rich tissues such as liver or whole fish. Therefore, in the
374 final procedure, we combined the cryo-precipitation at -20 °C with subsequent filtration of the
375 aqueous SPE medium. This simple three-step cleanup procedure resulted in rather satisfactory
376 matrix effects for muscle tissue, being in a range from 10% to -32% for all investigated analytes
377 (Fig. 3; Table S9). The signal suppression obtained by applying this procedure for the whole-fish
378 samples was somewhat higher but remained lower than 30% for 32 out of 42 analytes, respectively.
379

380 3.3. Method validation

381 The method validation included assessment of the linearity, instrumental detection limit (IDL),
382 method quantification limit (MQL), extraction recovery, accuracy, repeatability, and matrix effects
383 for two different matrices, surface water and fish tissues (Table S10, Table S11). Regarding the
384 final instrumental analysis, all analytes showed good linearity in the range from 1 to 500 µg/L with
385 an r^2 value higher than 0.996. Some analytes exhibited an extended linearity range down to 0.1
386 µg/L (i.e. verapamil, methadone, and terbinafine), whereas others showed increased linearity into
387 the higher concentration range up to 5 000 µg/L (i.e. torsemide, caffeine, valsartan). IDLs ranged
388 from 1.5 to 30 pg injected on column.

389 The key method parameters, accuracy and repeatability, were determined at three concentration
390 levels and the overview of the results for water and fish samples is given in Table 2. For water
391 samples, the accuracy was very good, spanning from 93% to 124%, 84% to 115%, and 84% to
392 116%, for concentration levels of 5 ng/L, 50 ng/L, and 500 ng/L, respectively (Table S10). The
393 repeatability, expressed as RSD of replicate sample analyses ($n = 4$), was very good at higher
394 concentration levels (mainly below 10%), and, as expected, somewhat higher RSD values were
395 obtained at the lowest concentration level, exceeding 20% for some analytes (morphine,
396 sulfamethoxazole, *N*-acetyl sulfamethoxazole). The method quantification limits of individual
397 analytes ranged from 0.1 to 2 ng/L, except for cotinine (5.5 ng/L), morphine (11.5 ng/L), and
398 norfloxacin (2.8 ng/L). Thus, the overall performance of the method for the analysis of water
399 samples is comparable to the LC/MS multiresidue methods published in the recent literature [37–
400 39], and can be considered fit for purpose for the determination of exposure concentrations of
401 selected drug residues at realistic conditions.

402 The method validation parameters, determined at three concentration levels (10 ng/g, 50 ng/g, and
403 250 ng/g) for the fish muscle and whole fish analysis are presented in Table S11. As for the water
404 samples, the accuracy of fish sample analysis was very high for all spiking levels (82% to 121%,
405 95% to 129%, and 82% to 112%, respectively), indicating the high robustness of the method. This
406 was achieved owing to the consistent use of isotopically labelled surrogates, which appears to be
407 superior to matrix matched calibration applied by Grabicová et al. [26]. Moreover, the method
408 repeatability at the two higher concentrations levels was typically from 4% to 16% for most
409 analytes, with only a few exceptions (RSD for *N*-acetyl sulfamethoxazole was 32%). Slightly
410 lower repeatability was obtained for the lowest concentration level, with some RSDs exceeding
411 20% (cotinine, codeine, oxcarbazepine, erythromycin).

412 Method quantification limits for biota samples were relatively low (0.1 – 5 ng/g), except for
413 cotinine (9.2 ng/g), morphine (26.7 ng/g), norfloxacin (6.5 ng/g) and erythromycin (4.3 ng/g),
414 which enabled the determination of trace concentration of selected drugs in feral fish samples.

415

416 3.4. Application to environmental samples

417 The total concentrations of the target analytes in the Sava River were from 10 809 to 10 888 ng/L
418 and from 7179 to 8844 ng/L, in autumn and spring, respectively. Of the total of 44 analytes, 43
419 and 30 of them were quantifiable in at least one of the collected water and biota samples,

420 respectively (Tables S12, S13, and S14). Amoxicillin was the only analyte that was not detected
421 in any of the collected water samples (<0.5 ng/L). The average concentrations for most of the
422 individual analytes in the collected Sava River water samples were in the ng/L range, from <0.1
423 to 562 ng/L and from <0.1 to 793 ng/L, in the autumn of 2021 and spring of 2022, respectively
424 (Fig. 4; Table S12). In both sampling periods, the highest concentrations were recorded for
425 macrolide antibiotic azithromycin, beta blocker valsartan, and analgesics tramadol with average
426 concentrations of 6.0 µg/L, 1.3 µg/L and 0.6 µg/L in autumn, and 1.9 µg/L, 0.9 µg/L and 0.8 µg/L
427 in spring. Other prominent contaminants, exceeding 100 ng/L, included sulfonamide
428 sulfamethoxazole and sulfapyridine, popular stimulant caffeine, antiepileptics carbamazepine and
429 oxcarbazepine, anxiolytic oxazepam as well as descladinosyl azithromycin, *N*- and *O*-desmethyl
430 tramadol, which are metabolites of the two most prominent drugs mentioned above. These
431 concentrations generally reflect their therapeutic consumption figures [3]. The concentrations for
432 most of the compounds in this work are comparable to those reported for the urban rivers [40–42],
433 except for azithromycin, which showed significantly higher levels in the Sava River. The reason
434 for such situation is a significant additional non-therapeutic input from the local pharmaceutical
435 industry as discussed in our earlier reports [6,43]. The concentration of AZI and its metabolites
436 was in the similar range as reported earlier for the secondary effluent of the city of Zagreb [43],
437 suggesting that the fish at this location has been chronically exposed to enhanced levels of
438 macrolide residues.

439 A summary of the results on the concentrations of the target drugs in fish muscle and whole fish
440 samples is given in Table 3, while the detailed results for each individual sample and analyte are
441 presented in Tables S13 and S14. The reasoning for the selection of muscle as target tissue relies
442 on the fact that it represents the edible part of the fish and thus may be relevant to estimate possible
443 human exposure to these contaminants via fish food. Similar approach has been included in the
444 EU Water Framework Directive [29] and related documents for the mandatory monitoring of
445 highly toxic priority contaminants. The muscle concentrations can also be used to assess the
446 bioaccumulation potential of drugs, but the current guidelines on this issue [44] recommend the
447 use of whole fish concentrations for this purpose. In fact, the concentration of individual drugs in
448 the fish muscle and whole fish in our samples were rather similar, varying from <0.1 to 39 ng/g
449 and <0.1 to 68 ng/g, respectively. The total concentrations of the target drugs were from 14 to 58
450 ng/g and 22 to 94 ng/g in muscle and whole fish samples, respectively. The most prominent analyte

451 found in biota samples were azithromycin (1.4 to 67.8 ng/g) and caffeine (0.5 to 18.6 ng/g), which
452 was detected in all collected fish specimens. The determined drug concentrations are in good
453 agreement with the data published in the literature [25,45]. The only result, which seems to be
454 highly specific for the Sava River, are consistently enhanced levels of azithromycin, which agrees
455 with the enhanced concentrations in water samples. The limited data set in this preliminary pilot
456 study does not allow the determination of possible subtle differences between the autumn and
457 spring seasons. Moreover, although the concentrations of some minor drugs revealed significant
458 variability between the individual fish specimens, there were no systematic differences between
459 different fish species. Consequently, more extensive studies will be required to address the issues
460 such as temporal variability of drug consumption, fish biometric characteristics and biological
461 cycles as well as possible species-specific differences related to trophic level and biomagnification.
462 To assess the bioaccumulation potential of our target compounds their corresponding
463 bioaccumulation factors (BAFs) were calculated by comparing the tissue concentrations with the
464 exposure concentrations in water. The detailed data for each individual fish specimen and target
465 drug are presented in Tables S15 and S16. The overview of the results is presented in Table 3
466 which includes only the drugs with measurable concentrations in both water and fish tissue
467 samples, for which BAFs could be reliably estimated. As compared to the current literature
468 [26,33,46] the results presented in Table 3 are in a similar order of magnitude. The BAFs in muscle
469 and whole fish are in similar ranges (from 0.2 to 431 L/kg and from 1 to 838 L/kg, respectively),
470 and some outliers can be explained by the fact that they were estimated for different fish
471 specimens. The highest BAFs, exceeding 100 L/kg, were observed for diazepam, sertraline,
472 lisinopril, torsemide, desloratadine, loratadine, and terbinafine. It is interesting to note that
473 azithromycin and caffeine, which showed high concentration levels in fish tissue, have a rather
474 modest bioaccumulation potential, with BAFs from 0.2 to 35 L/kg and from 1.4 to 27 L/kg,
475 respectively.

476 The data presented in this work revealed that, in general, pharmaceuticals do not belong to the
477 category of highly bioaccumulative compounds ($BAF > 1000$). Nevertheless, the recent report by
478 Malev et al. [47], based on chemical analysis of wild fish plasma samples from the Sava River,
479 revealed the widespread presence of pharmaceuticals and suggested, using the fish plasma model,
480 that several pharmaceutical classes might have a significant biological impact on fish at the current
481 exposure levels.

482

483 **4. Conclusions**

484 A wide-scope LC-MS/MS-based analytical method for the determination of multiclass drugs,
485 including the most common pharmaceuticals, drugs of abuse, and their metabolites was developed
486 to facilitate bioaccumulation assessment of these emerging chemicals in freshwater fish at
487 environmental conditions. The newly developed extraction procedure, based on UAE with basified
488 MeOH provided high extraction recoveries for all target compounds from fish tissue, while a
489 combination of cryo-precipitation, filtration of water-diluted UAE extract, and SPE ensured an
490 efficient reduction of the signal suppression. Thoroughly optimized and fully validated procedures
491 for water and fish tissue matrices proved highly reliable, robust, and suitable for routine high
492 throughput analyses. The application of the method in a polluted urban river system indicated that
493 most of the investigated drugs show relatively low BAFs, however several of them, including
494 lisinopril, sertraline, terbinafine, torsemide, diazepam, desloratadine, and loratadine were singled
495 out as potentially bioaccumulative substances.

496

497 **Ethical Approval**

498 All applicable institutional, national, and international guidelines for the welfare and use of
499 animals were followed. Electrofishing and fish collection were made with the authorization of the
500 Ministry of Agriculture of the Republic of Croatia (Class: UP/I-324-02/21-01/04 UP/I-324-01/22-
501 01/9, Reg. No: 525-13/0733-21-4 of November 3, 2021; and Class: UP/I-324-01/22-01/9, Reg.
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503

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509

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