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Bioaccumulation of abacavir and efavirenz in *Rhinella arenarum* tadpoles after exposure to environmentally relevant concentrations

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PII: S0045-6535(22)01124-9

DOI: https://doi.org/10.1016/j.chemosphere.2022.134631

Reference: CHEM 134631

To appear in: *ECSN*

Received Date: 4 February 2022

Revised Date: 7 April 2022

Accepted Date: 13 April 2022

Please cite this article as: Fernández, L.P., Brasca, R., Repetti, M.R., Attademo, André.M., Peltzer, P.M., Lajmanovich, R.C., Culzoni, Marí.J., Bioaccumulation of abacavir and efavirenz in *Rhinella arenarum* tadpoles after exposure to environmentally relevant concentrations, *Chemosphere* (2022), doi: https://doi.org/10.1016/j.chemosphere.2022.134631.

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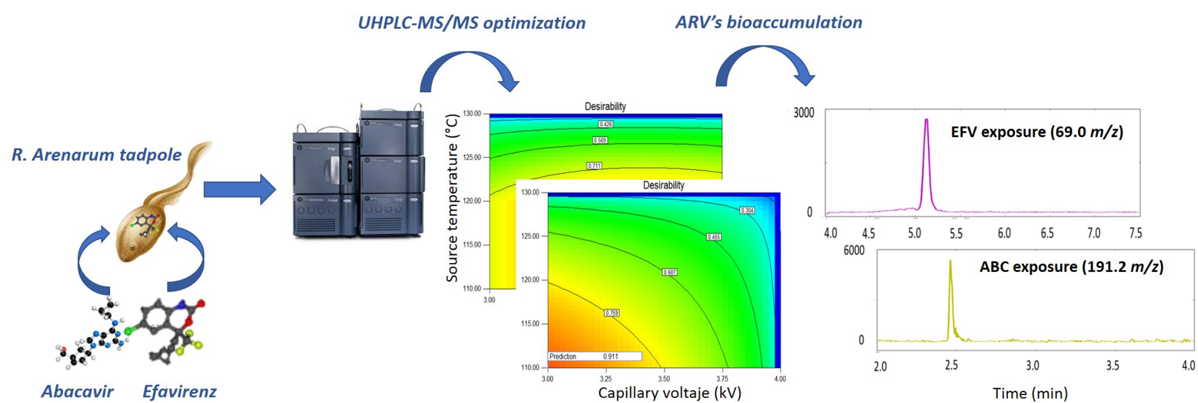

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1 # Bioaccumulation of abacavir and efavirenz in *Rhinella*
2 # *arenarum* tadpoles after exposure to environmentally
3 # relevant concentrations

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20 **Abstract**

21 Antiretrovirals are pharmaceuticals used in the treatment of the human immunodeficiency
22 virus; they are contaminants of emerging concern that have received considerable attention
23 in recent decades due to their potential negative environmental effects. Data on the
24 bioaccumulation and possible environmental risks posed by these drugs to aquatic
25 organisms are very scarce. Therefore, the aim of this study was to evaluate the
26 bioaccumulation of abacavir and efavirenz in *Rhinella arenarum* tadpoles subjected to
27 acute static toxicity tests (96 h) at environmentally relevant concentrations. The analytical
28 procedure consisted of the development and optimization of a method involving ultra-high
29 performance liquid chromatography with tandem mass spectrometry detection. The
30 instrumental conditions, optimized by design of experiments using the response surface
31 methodology, yielded limits of detection of 0.3 μg L^{-1} for abacavir and 0.9 μg L^{-1} for
32 efavirenz; and limits of quantification of 1.9 μg L^{-1} for abacavir and 5.6 μg L^{-1} for
33 efavirenz. Subsequently, the bioaccumulation of the pharmaceutical drugs in tadpoles was
34 evaluated at three exposure concentrations. Efavirenz displayed the highest
35 bioaccumulation levels. This study shows the bioaccumulation potential of abacavir and
36 efavirenz in amphibian tadpoles at exposure concentrations similar to those already
37 detected in the environment, indicating an ecological risk for *R. arenarum* and probably
38 other aquatic organisms exposed to these drugs in water bodies.

39

40 **Keywords:** Antiretrovirals; Emerging contaminants; Bioaccumulation; UHPLC-MS/MS

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41 **1. Introduction**

42 Contaminants of emerging concern comprise a wide variety of chemical compounds, 43 including pharmaceuticals and personal care products (PPCPs), nanomaterials, hormones 44 and steroids, surfactants, flame retardants, and microplastics, among others. Due to their 45 massive environmental release, they have been detected in air, soil and water through 46 sensitive analytical methods (Ramírez-Malule et al., 2020). They cause known or suspected 47 adverse ecological and/or human health effects, but are not usually included in routine 48 monitoring programs due to the lack of regulation criteria (Alcaráz et al., 2015; Ramírez-49 Malule et al., 2020; Valdés et al., 2016).

50 Among these emerging contaminants, which are consumed or used worldwide, the 51 environmental occurrence of PPCPs is a serious problem of health and environmental 52 concern of recent debate in the scientific literature. Antibacterial compounds, nonsteroidal 53 anti-inflammatory drugs, antivirals, steroid hormones and antidepressants are examples of 54 pharmaceutical drugs that have been detected in the environment (Omotola et al., 2022). 55 Particularly, the worldwide occurrence of antiviral drugs in wastewaters and natural waters 56 has been recently reviewed by Nannou et al. (2020), who indicated that the available data is 57 scarce and covers limited geographical regions. In fact, limited monitoring information was 58 gathered for several European and African countries, with even fewer data being available 59 for Asia and North America, and no data for other regions around the world, including 60 South America. Antiretroviral drugs were mainly investigated in African water bodies and 61 wastewaters. Both publications (Nannou et al. (2020) and Omotola et al. (2022)) indicate 62 that scarce data from ecotoxicity bioassays was available for antiviral drugs, and 63 international cooperation in research was recommended for prioritization of target drugs to

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64 be incorporated in monitoring studies. Moreover, ecotoxicity studies involving
65 pharmaceuticals were encouraged to increase the data bank.

66 Antiretroviral (ARV) drugs are pharmaceuticals used for treating the human
67 immunodeficiency virus (HIV). The treatment usually combines nucleoside analogue
68 reverse transcriptase inhibitors, such as abacavir (ABC), with non-nucleoside reverse
69 transcriptase inhibitors, such as efavirenz (EFV), in a fixed dose combination (Ncube et al.,
70 2018). Information related to the physicochemical properties of these ARVs is provided in
71 Fig. 1. These drugs are usually consumed daily by oral administration, and are excreted
72 from the body unchanged or partially metabolized, being discharged to the sewage system
73 (Abafe et al., 2018). In fact, ABC and EFV were detected in different types of water bodies
74 (wastewater influents and effluents, surface waters, groundwater and even drinking water)
75 at concentrations of 0.5 ng L^{-1} to 34 μg L^{-1} (Aminot et al., 2015; Funke et al., 2016; K'oreje
76 et al., 2012; Schoeman et al., 2017; Swanepoel et al., 2015), and in estuary sediments at
77 concentrations ranging between 0.1 and 3.0 μg kg^{-1} (Aminot et al., 2015; Rimayi et al.,
78 2018).

79 Several analytical methods, such as high performance liquid chromatography with
80 diode array detection (HPLC-DAD) (Paradina Fernández et al., 2020a; Pynnönen and
81 Tuhkanen, 2014), ultra HPLC with tandem mass spectrometry detection (MS/MS) (Mu et
82 al., 2016; Schoeman et al., 2017), pH-gradient flow injection analysis (FIA) with DAD
83 (Checa et al., 2006; Paradina Fernández et al., 2018) and, more recently, molecular
84 fluorescence spectroscopy (Paradina Fernández et al., 2020b), have been used for the
85 quantitation of ARVs in biological and environmental matrices. Since these
86 pharmaceuticals are found at low concentrations in the environment, the analytical
87 methodology should have high sensitivity, which can be achieved not only by analytical

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88 instrumentation, but also using chemometric tools, such as experimental design, to optimize
89 and maximize the responses of the target analytes (Paradina Fernández et al., 2020b).

90 The systematic presence of ARVs in aquatic environments may induce unexpected
91 effects in non-target organisms (K'oreje et al., 2012), such as liver damage and overall
92 health decline, as well as development and growth abnormalities in fish (Kowlaser et al.,
93 2022; Robson et al., 2017). Recent studies revealed the potential bioaccumulation of some
94 ARVs in tadpoles after short-term exposure (48 h) to sublethal concentrations, as well as
95 toxicological effects associated with the increase of the activity of the enzyme glutathione
96 S-transferase (Paradina Fernández et al., 2020a). Likewise, potential environmental risks
97 were reported in fish, daphnia and algae (Minguez et al., 2016; Ncube et al., 2018; Ngumba
98 et al., 2016; Robson et al., 2017). Particularly, ABC and EFV were detected in fish plasma
99 at levels of ng L^{-1}, indicating that these compounds tend to accumulate in aquatic
100 ecosystems (Swanepoel et al., 2015). However, information about bioaccumulation of
101 ARVs in aquatic species at environmental concentrations is scarce.

102 Bioaccumulation was indicated as an exposure-related parameter that should be
103 assessed in environmental risk assessment when appropriate (Feijtel et al., 1997). However,
104 bioaccumulation could be considered a hazard criterion itself, since some effects may be
105 detected only at a later life stage, and therefore affect several generations ; in other cases,
106 the effects are only manifested at higher trophic levels in the food web (Franke et al., 1994;
107 Valdés et al., 2016; van der Oost et al., 2003).

108 In this line, we worked under the hypothesis that ABC and EFV may pose potential
109 risks to the environment and, therefore, be candidates for future regulations and/or
110 monitoring programmes, especially in those regions of the world that are most affected by
111 HIV. Therefore, the aim of this study was to develop an analytical method (UHPLC-

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112 MS/MS) assisted by experimental design tools to evaluate the bioaccumulation of ABC and
113 EFV in *Rhinella arenarum* tadpoles after exposure to environmentally relevant
114 concentrations.
115

116 2. Materials and methods

117 *2.1 Reagents and solutions*

118 EFV and ABC were kindly supplied by Laboratorio DOSA S.A. (Buenos Aires,
119 Argentina). LC/MS-grade acetonitrile (ACN) and formic acid (FA) were purchased from
120 Fisher Scientific (New Hampshire, USA). HPLC grade methanol (MeOH) was acquired
121 from Biopack (Buenos Aires, Argentina). All reagents were of analytical grade. Ultrapure
122 water was obtained from a Millipore system (Bedford, USA).

123 ABC and EFV stock solutions of 500 μg mL^{-1} were prepared in ultrapure water and
124 MeOH, respectively, and stored in the dark at 4 ºC. Working solutions were prepared by
125 adequate dilution of the stock solutions to reach final concentrations of 2 μg mL^{-1}.
126

127 *2.2 Model test organisms*

128 *Rhinella arenarum* (Amphibia, Anura) tadpoles were used as model test organisms
129 because they generally take up contaminants through both their gills and skin, and are easy
130 to maintain under laboratory conditions. This anuran species is widely distributed in
131 tropical aquatic ecosystems (Cei, 1980) present in forests, wetlands, agricultural lands and
132 urban areas (Peltzer et al., 2017). In Argentina, this toad is considered "not threatened"
133 (Vaira et al., 2012) and is extensively distributed in the provinces of Buenos Aires,
134 Formosa, Chaco, Corrientes, Santiago del Estero, Entre Ríos and Santa Fe. Eggs were
135 collected from temporary ponds in natural floodplains of the Paraná River (31° 11' 31'' S,

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136 60° 9' 29'' W, Argentina) with collection permission of the Ministerio de Ambiente y
137 Cambio Climático, Santa Fe, Argentina (File N° 02101-0018518-1). This region is
138 considered to be non-polluted by pharmaceuticals and pesticide residues (Peltzer et al.,
139 2017). The eggs were transported to the laboratory, maintained in naturally dechlorinated
140 tap water (DTW) (pH = 7.4; conductivity = 165 μS cm^{-1}; dissolved oxygen = 6.5 mg L^{-1},
141 hardness = 50.6 mg L^{-1} $CaCO_3$ at 22 ºC), and allowed to develop until tadpoles reached
142 Gosner stages 26-28 (Gosner, 1960) to conduct the toxicity evaluation.
143
144 *2.3 Instrumentation and procedure*
145 The chromatographic experiments for ARV quantitation in tadpoles were performed
146 in an ACQUITY UPLC™ (Waters Corporation, Massachusetts, USA) equipped with a
147 binary pump, degasser membrane, auto-sampler, oven column compartment, coupled to
148 triple quadrupole mass spectrometer (MS/MS) equipped with a Z-spray orthogonal
149 ionization source (ESI) able to operate in positive and negative mode (TQD, Acquity
150 Micromass, United Kingdom). The individual and common parameters of the detection
151 system for each drug are described in Table 1. Data were analysed using the software
152 MassLynx version 4.1 (Waters, Manchester, United Kingdom).
153 The chromatographic separation was performed in an ACQUITY UPLC BEH Shield
154 RP 18 column (2.1mm × 100mm, 1.7µm particle size) (Waters Corporation, Massachusetts,
155 USA). The temperature column was set at 40 ºC and the flow rate at 0.4 mL min^{-1}.
156 Ultrapure water: ACN (98%:2%) and ACN (100%), both with 0.1% FA, were used as
157 mobile phase A and B, respectively. The UHPLC-MS/MS gradient was performed as
158 follows: 100% A and 0% B (0-1 min), 50% A and 50% B (1-3 min), 50% A and 50% B (3-
159 6.5 min), and finally, 100% A and 0% B (6.5-7.5 min). The injection volume was 10.0 µL.

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160 The chromatographic experiments for ARV quantitation in aqueous solution were
161 performed in an Agilent 1260 Infinity Ultra HPLC (UHPLC) system (Waldbronn,
162 Germany) equipped with a binary pump, degasser membrane, auto-sampler, oven column
163 compartment and UV-Vis diode array detector. OpenLab CDS Chemstation software
164 package (Agilent Technologies, Waldbronn, Germany) was employed for data acquisition
165 and analysis. The separation was performed in a Zorbax XDB-C18 column (4.6mm ×
166 75mm, 3.5-µm particle size) (Agilent, Waldbronn, Germany). The temperature column was
167 set at 25 °C and the flow rate was 0.8 mL min^{-1}. Ultrapure water and MeOH were used as
168 mobile phase A and B, respectively. The UHPLC gradient was performed as follows: 70%
169 A and 30% B (0-1min), 10% A and 90% B (1-8 min) and, finally, 10% A and 90% B (8-10
170 min). The total analysis time was 10 min, with 2 min for flushing the column and re-
171 establishing the initial conditions. The absorption wavelengths, which were used for
172 quantitation purposes, were 285 nm for ABC and 247 nm for EFV.
173
174 *2.4 Experimental design for the optimization of the MS/MS detection*
175 Experimental design tools were applied with the aim of optimizing the parameters
176 involved in the MS/MS detection and, therefore, allowing adequate sensitivity for both
177 ARVs. First, the factors that influence the MS/MS signals were determined by means of a
178 full factorial design (FFD) (Bezerra et al., 2008) involving six factors: capillary voltage (1
179 and 4 kV), extractor voltage (2 and 6 V), source temperature (120 and 150°C), desolvation
180 temperature (350 and 450°C), desolvation flow rate (800 and 1000 L h^{-1}) and cone flow
181 rate (5 and 20 L h^{-1}). A full factorial 2^{k-2} design was chosen, where k is the number of
182 factors to be evaluated at two levels (2^4 = 16 experiments). The areas under the
183 chromatographic peaks, corresponding to the transition of the product ions of highest

184 sensitivity (282.7→191.2 *m/z* for ABC and 314.0→69.0 *m/z* for EFV), were the selected
185 responses. An analysis of variance (ANOVA) was performed to define the factors that have
186 significant influence on the responses.

187 Subsequently, a central composite design (CCD) (Bezerra et al., 2008) with six
188 central points and alpha value equal to 1.68, compatible with rotatable distribution of
189 prediction variance, was built to investigate the effects of the selected variables and find the
190 optimal combination of the experimental conditions that produce the highest responses. For
191 each independent variable, five levels coded as −1, 0, and +1 (for low, medium, and high
192 levels, respectively), and – alpha and + alpha were analysed. Therefore, the investigated
193 levels were 3, 3.5 and 4 kV (alpha = 2.7 and 4.3 min) for the capillary voltage, 110, 120,
194 130 °C (alpha = 103 and 137 °C) for the source temperature, and 300, 337.5 and 375 °C
195 (alpha = 274 and 400 °C) for the desolvation temperature, and the fixed factors were the
196 desolvation flow rate (1000 L h^{-1}), the cone flow rate (5 L h^{-1}) and the extractor voltage (2
197 V) (see Table S1, Supplementary Material).

198 The evaluated responses were modelled using the Derringer's desirability function
199 (Eq. 1), a powerful strategy used for the simultaneous optimization of different objective
200 functions, i.e. responses (Myers et al., 2016).

201 $$D = ({d_1}^{r_1} \times {d_2}^{r_2} \times \ldots \ldots {d_n}^{r_n})\frac{1}{\Sigma r_i} = \left(\prod_{i=1}^{n} {d_1}^{ri}\right)^{\frac{1}{\Sigma ri}} \qquad (1)$$

202 where n is the number of variables included in the optimization procedure, d is the
203 individual desirability function of each response and r_n is the importance of each factor or
204 response relative to the others.

205

206 *2.5 Calibration samples*

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207 The calibration curves for the UHPLC-MS/MS method involved a five-sample
208 calibration set for ABC and a six-sample calibration set for EFV, and were built in triplicate
209 by transferring appropriate aliquots of 2 μg mL^{-1} working solutions to 5.0 mL volumetric
210 flasks and completing to volume with ACN:FA (99:1%). The final concentrations of the
211 calibration samples were within the range of 0.05 to 15.0 μg L^{-1} for ABC, and of 0.5 to
212 100.0 μg L^{-1} for EFV.

213 On the other hand, the calibration curve for the UHPLC-DAD method involved a
214 five-sample calibration set composed of mixtures of ABC and EFV, which was built in
215 triplicate by transferring appropriate aliquots of the working solutions to 5.0 mL volumetric
216 flasks and completing to volume with ultrapure water. The final concentrations of the
217 calibration samples were within the range of 10.0 to 125.0 μg L^{-1}.

218

219 *2.6 Acute static toxicity test*

220 Acute static toxicity tests (96 h) were conducted using sterile glass flasks (85 mm in
221 diameter, 110 mm in height) containing 200.0 mL of each ARV solution. The observation
222 periods and solution volumes were selected based on previous works (Attademo et al.,
223 2016; Lajmanovich et al., 2015; Paradina Fernández et al., 2020a; Svartz et al., 2012). The
224 laboratory conditions for the acute toxicity tests consisted of 12 h of light (>100 Lx) /12 h
225 of dark cycles at 22 ± 2 ºC with naturally DTW (pH = 7.4; conductivity = 165 µmhos cm^{-1};
226 dissolved oxygen = 6.5 mg L^{-1}; hardness = 50.6 mg L^{-1} $CaCO_3$ at 22 ºC). The temperature
227 and the irradiation/darkness cycles were selected to simulate the environmental conditions
228 (Quinn et al., 2011). The physicochemical properties of the DTW remained stable during
229 the whole toxicity test.

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230 In the present study, the acute static toxicity test was performed at environmentally
231 relevant concentrations, which were selected taking into account the ABC and EFV
232 concentration ranges already reported in aquatic environmental matrices (0.5 ng L^{-1} − 34 µg
233 L^{-1}) (Aminot et al., 2015; Funke et al., 2016; K'oreje et al., 2012; Schoeman et al., 2017;
234 Swanepoel et al., 2015). Larvae were exposed to ABC and EFV at nominal concentrations
235 of (0.5, 1.0 and 10.0 µg L^{-1}), and to a negative control containing only DTW. All solutions
236 were prepared with the same DTW used to raise the tadpoles. Each ARV treatment and the
237 negative control were performed in triplicate with five tadpoles per flask (n = 15). A total
238 of 105 tadpoles (average weight =0.026 ± 0.008 g) were used. Control and treated tadpole
239 were euthanized according to the criteria of ASIH (2011), and with the approval of the
240 animal bioethics committee of the Facultad de Bioquímica y Ciencias Biológicas,
241 Universidad Nacional del Litoral (Res. N°: 388/06). At the end of the experiment, i.e. after
242 96 h of exposure, each tadpole was gently blotted to remove excess water and weighed
243 using an electronic field balance. Each tadpole was placed individually in an Eppendorf
244 tube and preserved at −80 ºC until determination of drug bioaccumulation. In addition, 2.0
245 mL of each ARV solution were taken at the beginning and the end of the assay and stored
246 at –18 ºC until evaluation of ARV concentrations. It should be noted that due to the small
247 size of the tadpoles, the whole body was used for the bioaccumulation evaluation.
248
249 *2.7 Extraction method and matrix effect evaluation*
250 For the extraction of both analytes, each control tadpole was weighed, transferred to
251 an Eppendorf tube and spiked with the appropriate amount of each working solution to
252 evaluate three ARV concentrations (in triplicate) included in the calibration range, i.e. 1.0,
253 5.0 and 10.0 µg L^{-1} for ABC, and 5.0, 10.0 y 100.0 µg L^{-1} for EFV. Spiked samples were

254 kept at 4 ºC for 96 h until extraction. The extraction procedure was previously optimized
255 and described by Paradina Fernández et al. (2020a) (see Supplementary Material S1). The
256 whole procedure was performed in triplicate, and its performance was evaluated in terms of
257 percent recovery, as follows:

258 $$Recovery(\%) = \frac{Experimental\ ARV\ concentration}{Nominal\ ARV\ concentration} \times 100 \quad (2)$$

259 For the toxicity study, several pools were prepared using the five tadpoles per flask of
260 each exposure concentration, i.e. 0.5, 1.0, 10.0 µg L^{-1} for ABC, and 0.5 and 1.0 µg L^{-1} for
261 EFV. Each pool was reconstituted in 25.0 µL of ACN:FA (99:1%), except for the 10.0 µg
262 L^{-1} EFV exposure concentration, at which tadpoles were evaluated independently in a
263 reconstitution volume of 50.0 µL.

264 Additionally, a matrix effect evaluation was conducted in terms of suppression or
265 increase in signal intensity during the analysis of the samples by UHPLC-MS/MS. The
266 evaluated concentration levels were those described above. Thus, the slopes of the linear
267 regressions obtained for the spiked tadpole matrices and the pure standard solutions were
268 analyzed by means of a *Student t-test* (Eq. 3), considering the following hypothesis: H_0:
269 slope = 1 and H_1: slope ≠ 1.

270 $$t_{exp} = \frac{|1 - Slope|\sqrt{n}}{S_R} \quad (3)$$

271 where n is the number of samples ($n = 9$) and S_R is the standard deviation of the slope. The
272 slope is considered statistically different from 1 when the t_{exp} value exceeds the $t_{critical}$ value
273 (α, υ) for a given level α, $\upsilon = n - 1$ degrees of freedom and n samples (Olivieri, 2015).
274

275 *2.8 Pre-concentration method*

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276 A pre-concentration method for ABC and EFV quantitation in ARV solutions
277 through solid phase extraction (SPE), using Oasis®HLB cartridges 1cc (Waters
278 Corporation, Massachussets, USA), was developed (see Supplementary Material S2). The
279 percent recovery (Eq. 2) was evaluated at three exposure levels in triplicate, i.e. 0.5, 1.0 and
280 $10\,\mu g\ L^{-1}$.

281 The stability of the ARVs during the acute toxicity test was verified using this
282 method. For this purpose, solutions of the ARVs in DTW (without tadpoles), at the three
283 investigated levels, were subjected to the exposure cycles during 96 h. ABC and EFV
284 concentrations were measured at the initial and final exposure times. This assay was
285 conducted in triplicate and the comparison between both concentrations was assessed by
286 means of a *Student t-test* (Eq. 3).

287

288 *2.9 Data analysis and software*

289 Experimental designs, surface response modeling and desirability function
290 calculations were performed using the Stat-Ease Design-Expert 8.0.0 software (Stat-Ease,
291 Inc., Minneapolis, USA).

292

293 **3. Results and discussion**

294 *3.1 General considerations*

295 During the toxicity test, mortality was not observed either in the *R. arenarum*
296 tadpoles exposed to the ARV treatments or in the controls. In addition, no external signals
297 of toxicity, such as alteration in the swimming behavior, were observed. These results
298 provide valuable information to complement previous studies on bioaccumulation and
299 toxicological effects of ARVs in tissues of amphibian tadpoles during short-term exposure

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300 to sublethal concentrations. These vertebrates were recognized as indicators of early
301 warning to assess the quality of the environment, since they exhibit biological responses
302 under signs of ecotoxicity due to several emerging contaminants (Foster et al., 2010;
303 Peltzer et al., 2019; Veldhoen et al., 2014).

304

305 *3.2 Optimization of the MS-MS detection*

306 The achievement of the high sensitivity levels required for the quantitation of both
307 ARVs in tadpoles was ensured by combining chemometric tools and MS/MS detection. The
308 fundamental parameters involved in the ionization process of each analyte were firstly
309 screened by means of an FFD. Results of the ANOVA indicate that the capillary voltage,
310 the source temperature and the desolvation temperature were the significant factors
311 affecting the MS/MS signal intensity of the analytes.

312 Subsequently, with the aim of establishing the best combination of these three factors,
313 a CCD was conducted (see Table S1 of Supplementary Material). The experimental data
314 were fitted to a quadratic model using backward multiple regression, and the results were
315 examined using ANOVA with a significance level of $\alpha = 0.05$. The ANOVA test validated
316 the significance of the models for ABC and EFV (p =0.0181 and p=0.0191, respectively),
317 with the lack of fit being non-significant (p=0.9724 and p=0.8381, respectively). Then, the
318 responses were modelled using the Derringer's desirability function, following the
319 maximization criterion. In this sense, the experimental conditions corresponding to a
320 maximum in the desirability function (D = 0.911 for ABC and D = 0.853 for EFV) were the
321 capillary voltage at 3.00 and 3.66 kV, desolvation temperature at 400 and 340 °C, and
322 source temperature at 110 and 120 °C for ABC and EFV, respectively (Fig. 2). Therefore,

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323 these optimal conditions were employed to determine the absorbed ARV concentrations in
324 tadpoles.
325
326 *3.3 Extraction method and matrix effect evaluation*
327 The performance of the extraction method in terms of analyte recoveries for the
328 evaluated concentration levels were 90 ± 2% for ABC and 95 ± 3% for EFV. Therefore,
329 the procedure using ACN for direct protein precipitation proved to be suitable for the
330 simultaneous extraction of the analytes from the tadpoles. Besides, the absence of matrix
331 effect was evidenced with the t_{exp} values (0.68 for ABC and 0.01 for EFV), which were
332 lower than the $t_{critical\ (0.025,\ 8)}$ value of 2.31 in both cases.
333
334 *3.4 Evaluation of the pre-concentration method*
335 Average recovery values of 97±1 % for EFV and 94±3 % for ABC were obtained
336 after performing the SPE pre-concentration of the ARV exposure solutions, indicating the
337 suitability of the proposed sample pretreatment method.
338 The stability of both ARVs at the three concentration levels, analyzed by UHPLC-
339 DAD during the toxicity test, was confirmed through a *Student t-test* for a 95% confidence
340 level, with t_{exp} values of 0.02 and 0.19 for EFV and ABC, respectively, and $t_{critical\ (0.025,8)}$ =
341 2.31. Therefore, there was no variation of ABC and EFV concentrations under the
342 investigated experimental conditions.
343
344 *3.5 Determination of ABC and EFV in tadpoles and aqueous solutions*
345 To evaluate the performance of both analytical methodologies, several figures of
346 merit, such as sensitivity, analytical sensitivity, limit of detection and limit of

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347 quantification, were estimated according to Slutsky (1998). The results (Table S2,
348 Supplementary Material) confirm the excellent performance of the method regarding the
349 ARV quantitation under the evaluated conditions. As expected, the analytical sensitivity of
350 the UHPLC-MS/MS method was higher than that obtained for the UHPLC-DAD method;
351 consequently, lower limits of detection (0.3 μg L^{-1} for ABC and 0.9 μg L^{-1} for EFV) and
352 quantification (1.9 μg L^{-1} for ABC and 5.6 μg L^{-1} for EFV) were achieved.

353 The chromatographic profiles for the standard solutions of ABC (191.2 *m/z*) and EFV
354 (69.0 *m/z*), and for the extracts obtained from the tadpoles before and after being exposed to
355 each analyte individually are depicted in Figure 3. As expected, there was no significant
356 signal in the retention times of ABC and EFV in the control sample. On the contrary, ARV
357 signal contributions were observed in the chromatographic profiles associated with the
358 tadpoles exposed to the analytes, evidencing the bioaccumulation of ABC and EFV at the
359 evaluated exposure levels.

360 Table 2 summarizes the results of the concentrations absorbed by the tadpoles in the
361 acute static toxicity test. In both cases, ARV bioaccumulation increased with increasing
362 exposure level. Similarly, previous studies performed by our research group involving
363 toxicity tests for other ARVs reported that bioaccumulation increased at increasing
364 exposure concentrations (Paradina Fernández et al., 2020a). In addition, in the present work
365 EFV exhibited higher bioaccumulation than ABC, even at very low exposure levels. These
366 findings could be correlated with the high lipophilicity of EFV, which exhibited a log P
367 value of 5.4 (Fig. 1). In the case of ABC, a lower capacity of the drug to cross the lipidic
368 barrier of the tadpole skin can be suggested considering the informed permeability (log
369 P=1.6). Our results are consistent with those of Paradina Fernández et al. (2020a) found in
370 tadpoles exposed to lamivudine, stavudine, zidovudine and nevirapine. The authors

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371 observed different bioaccumulation rates between the ARVs, with nevirapine (log P = 3.9)
372 being more absorbed by tadpoles than the other drugs with lower lipophilicity. On the other
373 hand, our results are comparable to those of Mlunguza et al. (2020), who demonstrated a
374 higher uptake and bioaccumulation of EFV in aquatic plants than that of other ARVs
375 (emtricitabine and tenofovir disoproxil), which are less hydrophobic. Likewise, a higher
376 persistence and/or bioaccumulative behavior of EFV than that of other ARVs (acyclovir,
377 lamivudine and zidovudine) was reported for two species of aquatic organisms, the
378 cladoceran *Ceriodaphnia dubia* and the green algae *Raphidocelis subcapitata* (Almeida et
379 al., 2021). However, other possible mechanisms, such as differences in the
380 biotransformation rates and/or receptor-binding interactions, might be involved in the
381 accumulation of polar pharmaceuticals in aquatic organisms (Tanoue et al., 2014).

382 The bioconcentration factors (BCFs) were calculated as the ratio of ARV
383 concentration in tadpoles to the nominal ARV concentration in water. The obtained BCF
384 values, shown in Table 2, could be considered "non-bioaccumulative" according to the
385 Registration, Evaluation and Authorization of Chemicals (REACH) legislation in Europe
386 (Arnot et al., 2018). However, the BCF could be considered a parameter to estimate
387 metabolic biotransformation rates, rather than to predict potential bioaccumulation (Arnot
388 and Gobas 2006). In this work, higher BCF values were observed for EFV than for ABC,
389 suggesting a slower biotransformation rate for EFV than for ABC. Similarly, differences in
390 the bioaccumulation between aquatic species associated with the biotransformation rates
391 were reported for other emerging contaminants. For instance, in the case of triclosan, the
392 BCF values in algae and phytoplankton were higher than those found in invertebrate and
393 fish species, suggesting slower biotransformation rates for the smaller species (Arnot et al.,
394 2018).

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395 The results obtained in this study showed the accumulation of ABC and EFV in
396 tadpoles, even at low exposure levels (0.5 μg L^{-1}), especially of EFV. The scientific
397 literature related to the occurrence of ARVs in aquatic organisms is scarce and, to the best
398 of our knowledge, the presence of these drugs in fish was detected only in a few works. In
399 this sense, Swanepoel et al. (2015) detected ABC and EFV in blood plasma of *Clarias*
400 *gariepinus* at concentrations of 36 ng L^{-1} and 135 ng L^{-1}, respectively. Likewise, Robson et
401 al. (2017) informed hepatic damage that included steatosis and necrosis due to EFV acute
402 exposure of *Oreochromis mossambicus* to environmentally relevant concentrations. These
403 toxicological effects in aquatic species associated with EFV were evident at exposure
404 concentrations as low as 10.3 and 20.3 ng L^{-1}. Some authors proposed that the presence of
405 pharmaceuticals in the environment may produce effects on aquatic organisms similar to
406 the adverse effects reported for these drugs on humans (Arnold et al., 2013; Kolpin et al.,
407 2002; Robson et al., 2017). Therefore, the hepatic damage caused in fish could be
408 correlated with one of the main toxicity effects described in humans under EFV treatment,
409 i.e. hepatotoxicity (Tsibris and Hirsch, 2015). More recently, adverse effects associated
410 with physical abnormalities (including spinal deformities and alterations of the shape of the
411 jaws during morphological development of juvenile fish) were reported after EFV chronic
412 exposure of *Oreochromis mossambicus* to environmentally relevant concentrations
413 (Kowlaser et al., 2022).

414 On the other hand, toxic effects were recently reported in lettuce exposed to ARVs,
415 showing a high EFV bioaccumulation and subsequent physiological impact, associated with
416 the reduction in lettuce root and leaf biomass (Akenga et al., 2021). Although data
417 regarding the environmental risk assessment of these ARVs are scarce, some authors
418 reported that ABC was harmful to green algae *(Pseudokirchneriella subcapitata)* with EC_{50}

419 (half maximal effective concentration) values of 57 mg L^{-1} (Minguez et al., 2016; Nannou
420 et al., 2020). Likewise, potential toxic effects of EFV were indicated for *C. dubia* with EC_{50}
421 of 0.026 mg L^{-1}), and *R. subcapitata* with IC_{50} (50% inhibitory concentration) value of
422 0.034 mg L^{-1} (Almeida et al., 2021). Moreover, EFV was found to have mitotoxic effects
423 on both aquatic organisms, i.e., this drug triggered mitochondrial dysfunction, characterized
424 by direct inhibition of complex I of the electron transport chain, a decrease in the
425 consumption of oxygen, an increase in the production of reactive oxygen species and a
426 decrease in the potential of the mitochondrial membrane (Almeida et al., 2021; Apostolova
427 et al., 2017; Funes et al., 2014).

428 Hence, the results presented here, in agreement with the above-mentioned scientific
429 reports, demonstrate the potential toxicological effects of ARVs on non-target organisms,
430 indicating the environmental risk they can pose to aquatic organisms, particularly EFV, due
431 to its high bioaccumulation levels and possibly associated harmful effects. It should be
432 noted that our study evaluated ARVs at the levels expected to be found in the environment,
433 since, in the case of EFV, concentrations up to 34 μg L^{-1} were reported in aquatic
434 environments (Abafe et al., 2018; K'oreje et al., 2016; Schoeman et al., 2017). In addition,
435 the capacity of EFV to persist in aquatic environments, with very low removal rates by
436 wastewater treatment plants (WWTPs)(Abafe et al., 2018), indicates its potential to enter
437 the aquatic environment through WWTP effluents (Nannou et al., 2020; Ncube et al.,
438 2018).

439 It is evident that the consequences of the presence of EFV in surface waters are of
440 concern. This research evaluated tadpoles after ARV acute exposure for 96 h. However, the
441 aquatic organisms might be chronically exposed to these drugs during their life cycle,
442 which could pose an environmental risk that should be further investigated through chronic

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443 toxicity tests as well as through toxicological effect studies such as genotoxicity and other
444 ecotoxicological risk biomarkers. Biological end-point responses such as growth,
445 reproductive potential and survival/mortality could be also evaluated.

446 The results of the evaluation of ABC and EFV concentrations in the aqueous ARV
447 solutions are shown in Table 3. As expected, the concentrations obtained before exposure
448 (indicated as t = 0 h) were similar to the nominal concentrations for both ARVs. Likewise,
449 the concentrations obtained after the period of evaluation (indicated as t = 96 h) for the
450 control ARV solutions (without tadpoles) were similar to the initial concentrations,
451 demonstrating the stability of these drugs during the acute static toxicity test. On the other
452 hand, the concentrations of ABC and EFV in water at the final time of exposure in the
453 presence of tadpoles were lower than the initial concentrations (non-exposure), with the
454 percent decrease ranging between 13-19% for ABC and 31-43% for EFV. As can be
455 observed, the higher the exposure levels, the lower the concentrations of both analytes in
456 water at 96 h, which is in good agreement with the results of bioaccumulation in tadpoles.
457 Likewise, the reduction of EFV concentration in water was higher than that of ABC
458 concentration, in agreement with the results indicated in Table 2.

459 Importantly, EFV and ABC did not bioaccumulate in the tadpoles in the same ARV
460 uptake proportion due to expected differences in uptake, biotransformation and elimination
461 rates. In this sense, the drugs could be excreted from the tadpoles both unaltered (parent
462 compound without any biochemical transformation) and/or as metabolites, which should be
463 even more easily excreted due to the conversion of the drug into a more hydrophilic
464 compound (van der Oost et al., 2003).

465 It is known that many environmental contaminants induce oxidative stress in the
466 evaluated organisms, activating detoxification mechanisms that allow the excretion of these

467 drugs from the body. For example, the antioxidant defense mechanism induces antioxidant
468 enzymes involved in conjugation reactions; thus, the elimination of drugs and their
469 metabolites is facilitated through the increase of their solubility and the subsequent
470 transport of the modified compounds out of the cells (Ku et al., 2018; Martínez-Guitarte,
471 2018). Several studies involving the exposure of aquatic organisms to emerging
472 contaminants demonstrated significant increases in antioxidant enzymes as a strategy to
473 ameliorate oxidative stress (Oliveira et al., 2015; Pan et al., 2018; Peltzer et al., 2019,
474 2017). Likewise, it was demonstrated that tadpoles exposed to several ARVs in static acute
475 toxicity tests (48 h) at sublethal concentrations exhibited increased glutathione S-
476 transferase activity as compared to the control group, indicating potential oxidative stress
477 damage (Paradina Fernández et al., 2020a). These detoxification mechanisms allow the
478 induction and inhibition of several enzymes and proteins closely associated with the
479 metabolism and accumulation of the target chemical compound in organisms (Ku et al.,
480 2018). The activation of detoxification pathways to decrease bioaccumulation in aquatic
481 organisms, as a consequence of the exposure to emerging contaminants, is likely a crucial
482 evolutionary strategy of the defense mechanisms.

483 Despite the evidence of detoxifying mechanisms to respond to emerging
484 contaminants in amphibians, in this study, the observed bioaccumulation levels were
485 significant, especially for EFV, taking into account the low exposure concentrations
486 evaluated. Therefore, considering that the bioaccumulation of emerging contaminants in
487 biota may be a pre-requisite for adverse effects on ecosystems (Franke et al., 1994; van der
488 Oost et al., 2003), the presence of these ARVs in aquatic ecosystems poses an
489 environmental risk related to potential ecotoxicological effects that could be triggered in
490 aquatic organisms both in short- and long-term exposure.

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491 **4. Conclusions**

492 We determined the bioaccumulation of EFV and ABC in *R. arenarum* tadpoles at
493 environmentally relevant exposure concentrations. An analytical methodology achieving
494 high sensitivity levels was developed and optimized by means of experimental design.
495 Therefore, the applicability of this tool through the response surface methodology for
496 analytical signal optimization, minimizing the number of experiments to be conducted, the
497 solvent consumption and reducing the experimental time as compared to non-chemometric
498 assisted optimizations, was reinforced. The results evidenced the potential bioaccumulation
499 of these ARVs, particularly EFV, in *R. arenarum* tadpoles. The high bioaccumulation
500 levels detected for EFV should be a warning to the scientific community, considering that
501 the evaluated exposure concentrations are those found in real environmental scenarios. This
502 fact indicates the potential of this contaminant of emerging concern to be absorbed by non-
503 target organisms and bioaccumulate in a few days.

504 **Acknowledgements**

505 We thank Laboratorio DOSA S.A. for supplying the ARV drugs, Universidad Nacional del
506 Litoral (Projects CAI+D 2016-50120150100110LI and 50020150100063LI), CONICET
507 (Consejo Nacional de Investigaciones Científicas y Técnicas, Project PIP-2015 Nº 0111)
508 and ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica, Projects PICT
509 2014-0347 and PICT 2014-0470) for financial support. LPF is a doctoral fellow at
510 CONICET.

511 J. Brasca revised the English style.

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752 Table 1: MS/MS parameters used for the detection of ABC and EFV.

Parameters	**ABC**	**EFV**
Ionization mode	Positive (+)	Negative (-)
Capillary voltage (kV)	3.00	3.66
Source temperature (^{0}C)	110	120
Desolvation temperature (^{0}C)	400	340
Desolvation flow rate (L h^{-1})	1000	
Cone flow rate (L h^{-1})	5	
Extractor voltage (V)	2	
Retention time (min)	2.47	5.12
Precursor ion (*m/z*)	287.2	314.0
Dwell (s)	0.1	0.5
Cone (V)	25	35
Product ion 1 (*m/z*)	150.2	69.0
Collision energy (eV)	30	30
Product ion 2 (*m/z*)	174.2	244.0
Collision energy (eV)	31	20
Product ion 3 (*m/z*)	191.2	-
Collision energy (eV)	20	-

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755 Table 2. ARV concentrations absorbed by tadpoles at environmentally relevant exposure
756 levels.

Nominal concentration in water (µg L^{-1})	ABC [a]		EFV [a]	
	Concentration	BCF [b]	Concentration	BCF [b]
Control [c]	ND [d]		ND [d]	
0.5	0.042 (6) [e]	0.08 (3)	3.3 (6)	4.1 (2)
1.0	0.097 (7) [e]		4.7 (7)	
10.0	0.45 (6)		36 (2)	

757 Concentrations measured after 96h of exposure
758 [a] All the values are expressed as the average of the corresponding replicates in ng g^{-1}. Experimental standard
759 deviations are shown in the last significant figure in parentheses.
760 [b] BCF: Average bioconcentration factor (L kg^{-1}).
761 [c] Control: Tadpoles not exposed to ARV.
762 [d] ND: Not detectable.
763 [e] Detectable but not quantifiable.
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767 Table 3. ARV concentrations measured in water at initial (t = 0 h) and final (t = 96 h)
768 exposure times.

Nominal concentration in water ($\mu g\ L^{-1}$)		ABC		EFV	
		Measured concentration in water ($\mu g\ L^{-1}$)			
		t=0h	t=96h	t=0h	t=96h
Control [a]	0.5	0.48	0.46	0.49	0.50
	1.0	0.95	0.90	0.97	0.94
	10.0	9.55	9.64	9.66	9.69
ARV solution (with tadpoles)	0.5	0.46	0.40	0.48	0.33
	1.0	0.95	0.79	0.97	0.64
	10.0	9.10	7.40	9.60	5.51

769 [a] Control: Control ARV solution (without tadpoles) for each exposure level.
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772 **Figure captions**

773 **Figure 1.** Chemical structure and physicochemical properties of ABC and EFV.

774 **Figure 2.** Response surface of ABC (A) and EFV (B) for desirability as a function of the
775 studied factors for signal intensity (peak area) maximization criteria.

776 **Figure 3.** Chromatographic profiles for the product ion of ABC (191.2 *m/z*) and EFV (69.0
777 *m/z*) standard solutions (5 µg L^{-1} for ABC and 50 µg L^{-1} for EFV), and the extracts
778 obtained from tadpoles before (control) and after exposure to each ARV.

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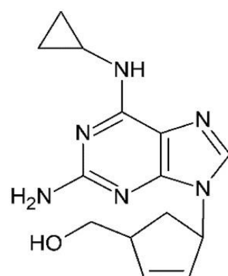
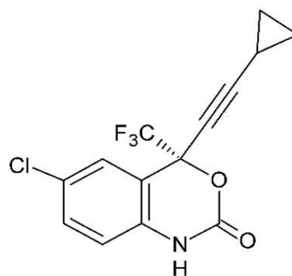
Figure 1



Abacavir (ABC)

Molecular weight [a]: 286.3 g mol^{-1}
pK_a= 5.2 [b]
Log P=1.6 [c]



Efavirenz (EFV)

Molecular weight [a]: 315.7 g mol^{-1}
pK_a= 10.1 [b]
Log P=5.4 [d]

[a] (PubChem)
[b] (Paradina Fernández et al., 2018)
[c] Octanol-water partition coefficient of the drug in its uncharged form calculated using KowWin® software (Fick et al., 2010)
[d] (Usach et al., 2013)

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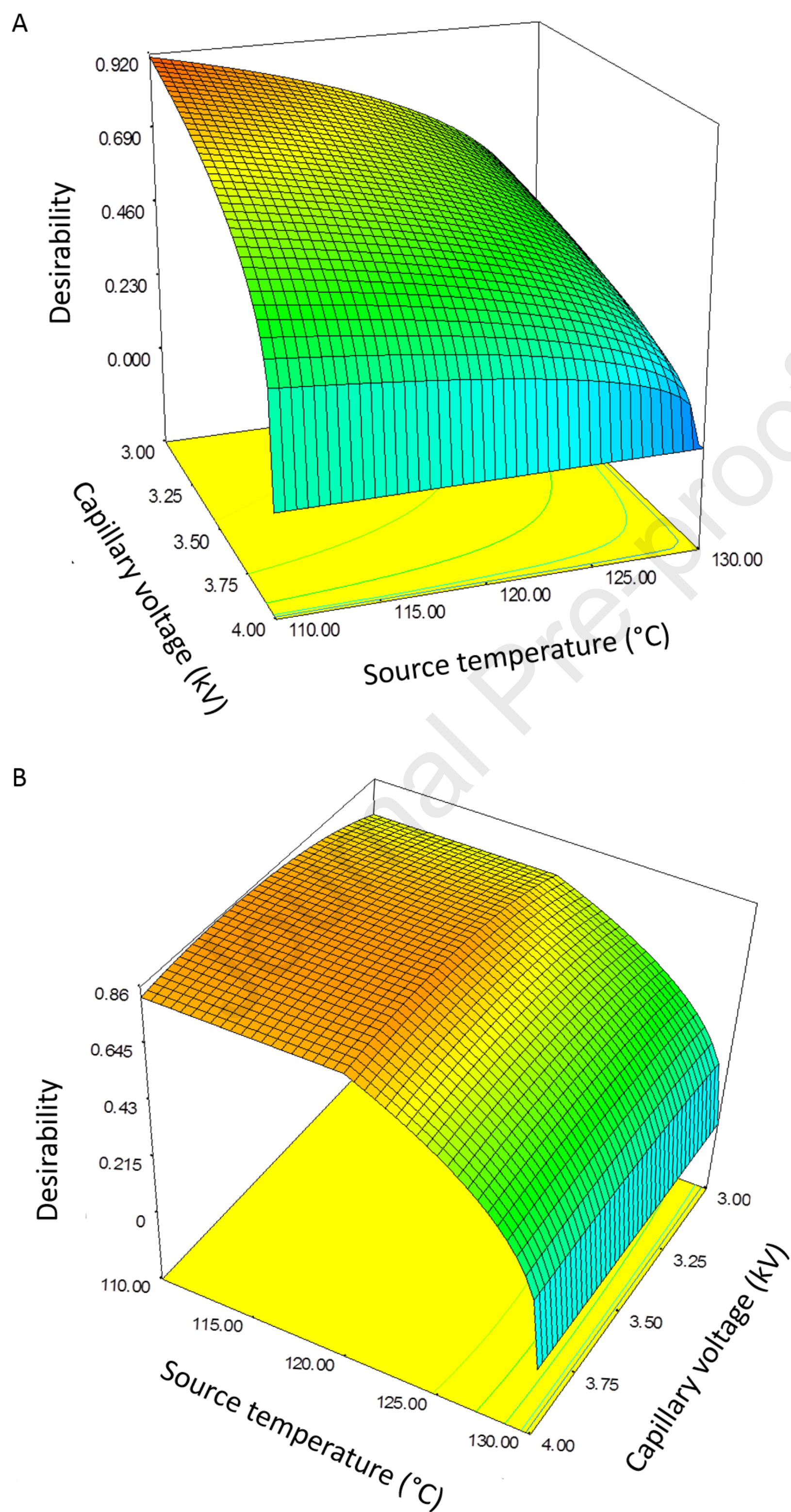
801 **Figure 2**



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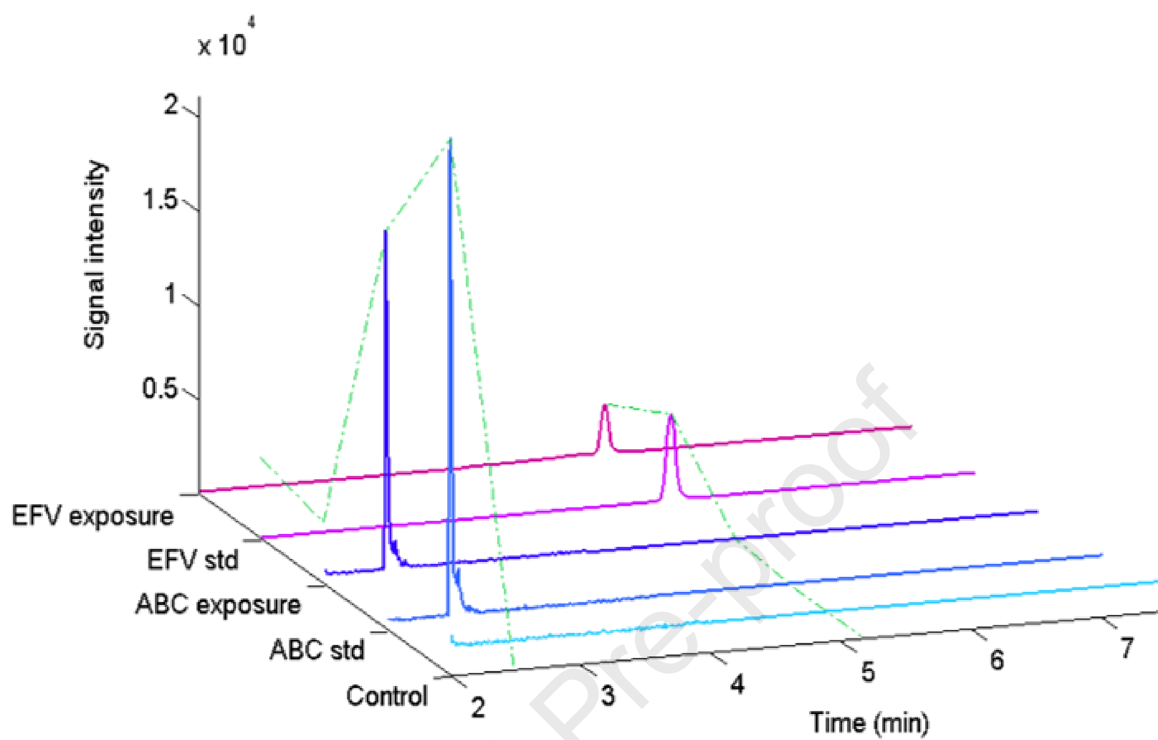
803 **Figure 3**



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Highlights

- Assessment of bioaccumulation of the antiretrovirals abacavir and efavirenz on *Rhinella arenarum* tadpoles
- Development of acute static toxicity tests (96 h) at concentration levels expected to be found in the environment
- Development and optimization of a method involving UHPLC-MS/MS
- Optimization of the MS detection to reach high sensitivity by Design of Experiments

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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