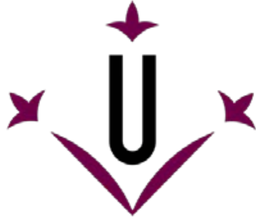

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Bioaccessibility of ochratoxin A from red wine by an *in vitro* dynamic gastrointestinal model

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Abstract

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus* and *Penicillium* species with immunosuppressive, teratogenic and carcinogenic properties. It has been determined that wine is the second source of OTA (10% of total OTA intake) in the European diet, and that its presence, even in small doses, can be a problem for long-term toxicity. In this paper we evaluated the bioaccessibility of OTA in a spiked red wine, in fasting human conditions, using an *in vitro* dynamic digestion model that includes a continuous-flow dialysis system to simulate the intestinal passage. To our knowledge, this is the first report on bioaccessibility of OTA in wine. A liquid-liquid method was used for the extraction of the OTA and ochratoxin alpha (OTα) from the gastrointestinal juices, and the extracts were analyzed by HPLC-FD. The bioaccessibility of OTA from the spiked red wine (1.0, 2.0 and 4 μg/L) was high in the gastric compartment (102.8%, 128.3% and 122.3%, respectively), whereas in the simulated intestine it did not exceed the 26%, and the OTA that crossed the dialysis membrane was very low (< 3.3 %). The amount of OTα in gastric chyme ranged from 5.1-19.1% of the spiked OTA, whereas in the intestinal compartment it did not exceed the 5%. As conclusion, in the *in vitro* system assayed, OTA showed a high bioaccessibility in the simulated stomach, but it decreased after the intestinal digestion and the passage through the dialysis membrane.

Keywords: bioaccessibility, *in vitro* digestion, mycotoxins, ochratoxin alpha.

78 **1. Introduction**
79
80 Ochratoxin A (OTA) is a toxic secondary metabolite naturally produced by some fungal species
81 belonging to the *Aspergillus* and *Penicillium* genera. OTA is a potent nephrotoxin (IARC 2B
82 group) that also exhibits immunosuppressive, teratogenic, and carcinogenic properties. OTA can
83 be found in different raw materials (such as cereals and fruits) and foodstuffs, such as grapes and
84 wine (IARC, 1993). Several studies have reported that the OTA amount in wine can be reduced
85 but not fully degraded or extracted during the main stages of wine making, either in the
86 fermentation process (Cecchini *et al.,* 2006; Esti *et al*., 2012), wine ageing (Anli *et al.,* 2011), or
87 through physical treatments, e.g., wine clarification and decontamination with adsorbents
88 (Castellari *et al*., 2001; Var *et al.,* 2008).
89
90 Due to the adverse effects that OTA can have on health, the Commission Regulation 1881/2006
91 sets a maximum level of 2 µg of OTA/L for wine and 10 mg of OTA/kg for dried vine fruit
92 (currants, raisins, and sultanas) (EC, 2006a). Although OTA is usually detected in wine at levels
93 below 1 µg/L, concentrations exceeding the legal limits are found with frequency (EC, 2002),
94 which is worrisome considering that wine is the second most important source of OTA (13-10%
95 of the total intake) in the European diet, after cereals (EC, 2002) and that wines are the second
96 most consumed alcoholic drink after beer (WHO, 2013).
97
98 It is known that OTA exposure is mostly by ingestion, however, a known intake of mycotoxins
99 through food does not always reflect the amount of toxin that is able to cross the intestinal
100 membrane. Some *in vitro* models have been developed to determine the amount of contaminant
101 released (bioaccessible fraction) from food during the gastrointestinal passage. Release of the
102 toxin from food, in the gastrointestinal tract, is a prerequisite for uptake (and bioavailability) in
103 the body. The bioaccessible fraction is defined as the amount of free contaminant that may
104 become available for absorption, since it has been released from the matrix and solubilized in the
105 gastrointestinal fluid (Versantvoort *et al*., 2004). On the other hand, ochratoxin alpha (OTα) is a
106 derivative from OTA formed in the gastrointestinal tract when carboxypeptidase A and
107 chymotrypsin break inside on the peptide bond and carboxyl-end, respectively, of OTA
108 (Kumagai and Aibara, 1982).
109
110 To date, no bioaccessibility studies of OTA have been developed in wine, a beverage that can be
111 usually consumed during long periods of adult life. The aim of this study was to estimate the
112 bioaccessibility of OTA in spiked red wine through a dynamic *in vitro* gastrointestinal model
113 simulating the digestion process, as well as the formation of OTα in the gastrointestinal juice.
114
115 **2. Materials and methods**
116 *2.1. Chemicals, reagents, and equipment*
117
118 Ochratoxin A (OTA) (CAS: 303-47-9) (purity 98%) was supplied by Sigma-Aldrich (St. Louis,
119 MO, USA), whereas ochratoxin alpha (OTα) (CAS: 16281-39-3) was from Romer Labs
120 Diagnostic (Tulln, Austria). All salts used to prepare the gastrointestinal solutions and the
121 hydrochloric acid (HCl, 37%) were supplied by Panreac, Chemical S.A. (Barcelona, Spain) or
122 Fisher Bioreagents (Fair Lawn, NJ). Orthophosphoric acid and sodium sulfide were from
123 Scharlau Chemical S.A. (Barcelona, Spain). Organic solvents and HPLC grade reagents were
124 from Fisher Scientific (Leicestershire, UK). The simulated digestive system was designed with

125 an adapted Liebig-West condenser, endfitting fluid connectors, silicone tubing (12 mm diameter)
126 and dialysis tubing cellulose membrane from Sigma-Aldrich (D9277) with a molecular mass cut-
127 off of 12400 Da and 32.8 mL/m of capacity (10 mm of flat width x 6 mm of diameter). A D-
128 21FT peristaltic pump from Dinko Instruments (Barcelona, Spain) and a thermostat bath
129 Tectron-bio-100 from J.P. Selecta, S.A. (Barcelona. Spain) were also employed.
130
131 2.2. *Wine*
132
133 In this study, we used a commercial Spanish red wine made from a Tempranillo grape variety
134 harvested in 2011 from the Castilla-La Mancha region. The initial OTA and OTα contamination
135 in this wine was evaluated using the method described by Muñoz *et al*. (2009). In-house
136 validation of the method was done at three OTA and OTα contamination levels (1.0, 2.0, and 4.0
137 μg/L) to determine the recovery of the toxins from the matrix. Recoveries ranged from 92.4 to
138 100.0% (RSDr 1.30-11.54%) for OTA and 63.7-66.9% (RSDr 15.9-20.1%) for OTα. The amount
139 of OTA found in this wine, considering the mean recovery of the method, was 0.103 μg/L,
140 whereas, as expected, no OTα was detected. This amount of OTA was taken into account for
141 bioaccessibility calculation.
142
143 *2.3. In vitro digestion procedure*
144
145 The digestion model used was a slightly modified version of the technique developed by Gil-
146 Izquierdo *et al*. (2002), including a continuous-flow dialysis system to simulate the intestinal
147 passage. The model describes a three-step procedure mimicking the digestive process in the
148 mouth, stomach (gastric digestion), and small intestine (intestinal digestion). The constituents
149 and concentrations of the simulated saliva, gastric juice, duodenal juice and bile juice employed
150 were those described in Versantvoort *et al*. (2005). Digestion assays were carried out with 5 mL
151 of red wine (spiked at 1.0, 2.0, or 4.0 μg OTA/L, i.e., with 5, 10 and 20 ng OTA) and the gastric
152 and intestinal mixtures were incubated for 2 h. The intestinal mixture was introduced into the
153 dialysis tube in a continuous-flow dialysis system simulating the intestine.
154 The dialysis system was designed with an adapted Liebig-West condenser and end-fitting fluid
155 connectors. The first chamber contained the dialysis tube, through which the intestinal mixture
156 flowed during the intestinal digestion step by using a peristaltic pump with a flow rate of 1
157 mL/min, and a phosphate buffer solution (PBS, pH 7.4) that bathed the dialysis tube. A
158 temperate water solution was pumped from a bath through a water jacket to keep the system's
159 temperature constant at 37 ºC (Figure 1).
160 Once the gastric and intestinal digestions completed, the fluids (gastric, intestinal, and the
161 dialysate) were collected and analyzed. The fluids were centrifuged for 10 minutes at 100 rpm at
162 10 ºC, yielding a supernatant that was analyzed for OTA and OTα content. Overall, for each
163 spiking level, six parallel experiments were carried out, from which three were interrupted for
164 analysis after the gastric phase, and the remaining three underwent the intestinal phase before
165 their analysis.
166
167 *2.4. Sample analysis*
168 *2.4.1. Liquid-liquid extraction of OTA and OTα from the simulated physiological fluids*
169
170 The extraction of OTA and OT*α*, was done using the method described by Muñoz *et al*. (2009),
171 with slight modifications. The gastric chyme was diluted 1:1 with $NaHCO_3$ (1%), and the pH

172 adjusted to 3 with 6 M H_3PO_4; the intestinal chyme and dialysis samples were diluted 1:4 in
173 H_3PO_4/NaCl (0.5 M:2 M) and the pH was adjusted to 1.6 using 10% $NaHCO_3$ (Zimmerli and
174 Dick, 1995). The organic phase was recovered and dried under a gentle stream of nitrogen at 40
175 ºC. Samples were stored at 4 ºC until HPLC analysis. To determine if the physiological fluids
176 caused chromatographic interference, two digestions free from wine were performed following
177 the previously described digestion procedure. After the incubation period for each digestion
178 phase, the juices were extracted directly or previously spiked with OTA and OTα (at 1.0, 2.0,
179 and 3.0 μg/L), and the toxin was immediately recovered using the described liquid-liquid
180 extraction method (Figure 2).
181
182 *2.4.2. High Performance Liquid Chromatography (HPLC) analysis*
183
184 The HPLC analysis of OTA and OTα was done using a Waters 2695 Separations Module
185 coupled to a Waters 2475 Multi λ fluorescence detector. The integration software used to manage
186 the chromatographic data was Empower 2 (2006 Waters Corporation, Database Version
187 6.10.00.00). Retention times were 10 min (OTα) and 27 min (OTA). Figure 2 shows the
188 chromatograms of the extracted physiological fluids (spiked or unspiked with OTA and OTα)
189 and a sample of the spiked wine post-digestion. Method performance was evaluated in terms of
190 linearity, limit of detection, limit of quantification, inter-day repeatability, and recovery rates.
191
192 *2.5. Statistical analysis*
193
194 Validation to estimate inter-day repeatability was performed; runs were done by triplicate.
195 Results are shown as the means ± standard deviations. Analysis of variance (ANOVA) was used
196 to test if there were significant differences ($p< 0.05$) in the calculated bioccessibility and for the
197 values of mycotoxin that cross the dialysis membrane, at the various tested OTA concentrations.
198

199 **3. Results and discussion**

200 *3.1. Method validation for OTA and OTα* analysis
201
202 The detection (LOD) and the quantification (LOQ) limits for the two toxins were experimentally
203 determined from the calibration curve of a set of two-fold serial dilutions of the toxin standards
204 (from 100 to 0.006 ng/mL), which was linear in the range from 0.012 to 100 ng/mL (r^2=0.995).
205 The LOD and LOQ determined for OTA were 0.012 and 0.036 ng/mL, whereas for OTα these
206 were 0.024 and 0.072 ng/mL, respectively. The method was repeatable for the two toxins at the
207 three assayed spiking levels, with a relative standard deviation (RSDr) bellow 5% in most cases.
208 OTA and OTα recoveries from the dialysis fraction were similar and most times above 80%, but
209 in the gastric and intestinal fractions recoveries were lower for OTα (62-78%), although the
210 RSDr percentages were similar to those obtained with OTA (Table 1). OTA recoveries fit the
211 performance criteria established in the EU Regulation 401/2006 (EC, 2006b) for the official
212 control in foodstuffs. Although there are no established performance criteria for OTα, all the
213 recovery percentages were homogeneous at the three spiking levels. The bioaccessibility results
214 and the amount of mycotoxin that crossed the dialysis membrane were corrected considering the
215 recovery rates.
216
217 *3.2. Bioaccessibility of OTA in* wine

218 The extractable compounds from the used physiological fluids did not interfere with the
219 chromatographic analysis of OTA (Figure 2). In our study, OTA levels detected in the gastric
220 chyme were quite high, representing a bioaccessibility of 102.81, 128.26, and 122.25% in each
221 of the tested contamination levels (Table 2). No significant differences were determined for the
222 three bioaccessibility values ($p<0.05$). Our results show that in the tested OTA concentration
223 range, the food matrix did not affect the bioaccessibility of OTA in the gastric chyme.
224
225 High bioaccessibility levels (86 to 116%) were determined by Versantvoort *et al*. (2005) in
226 buckwheat, peanuts, and mixtures of these cereals (4, 11, 51 ng OTA in the digestion model).
227 Avantaggiato *et al*. (2007) also described a high bioaccessibility of OTA (88%) from the
228 stomach to the small intestine, in a corn food at only one contamination level (11 μg OTA/kg).
229 Our results and the results of studies mentioned above are in line with the *in vivo* data reported
230 by Galtier (1978) and Roth *et al*. (1988), authors that attribute this behaviour to the acidic
231 properties of OTA (Galtier, 1978; Kumagai and Aibara, 1982). However, Kabak *et al*. (2009)
232 reported much lower bioaccessibilities; only 22% was found for buckwheat (24.9 μg OTA/kg)
233 and 29-32% for infant food (2-13.1 μg OTA/kg), in spiked foods.
234
235 In our study, during intestinal digestion OTA levels decreased, giving bioaccessibilities ranging
236 from 21 to 26%, while less than 3.3% was detected in the intestinal dialysate (Table 2). Glahn *et*
237 *al*. (1996) and Jovaní *et al*. (2001) also observed this behaviour, where the compounds that form
238 complex with proteins, or with mixed bile salt micelles, as OTA, are likely to be less
239 bioaccessible.
240
241 Results of *in vivo* experiments showed that OTA could have highly variable bioavailability
242 values. For example, the highest bioavailability was reported in mouse (92%), followed by pigs
243 (65.7%), rats (61%), monkeys (57%), rabbits (55.6%), chickens (40%) and fish (1.6%) (Galtier
244 and Alvinerie, 1981; Hagelberg *et al*., 1989). Unlike of the *in vitro* systems, it has to be noted
245 that *in vivo* the absorption in proximal jejunum depends of gastric emptying, mechanical
246 movements and can take place against a concentration gradient and depends on the pH at the
247 mucosal surface of the jejunum Kumagai, 1988; Kumagai and Aibara, 1982).
248
249 In our case, the bioaccessibility did not depend on initial mycotoxin levels ($p<0.05$), neither at
250 the gastric nor at the intestinal phase. However, with other mycotoxins, differences in
251 bioaccessibility could also be related to the way the matrix (food or feed) is contaminated
252 (spiked or naturally), and the bioaccessibility model employed for studying the release of
253 mycotoxins from the matrix. Two *in vitro* models to study mycotoxin bioaccessibility have been
254 primarily used by different authors: 1) the RIVM model (Versantvoort *et al*., 2004) and 2) the
255 Gil-Izquierdo model (Gil- Izquierdo *et al*., 2002). A third model, the TIM-1 model, has been
256 mainly employed in bioaccessibility assays of mycotoxins from cereals or food (Avantaggiato *et*
257 *al*., 2003, 2004, 2007; Zeijdner *et al*., 2003) in porcine gastrointestinal tract conditions. In-depth
258 review of the conditions and digestion models was done in our previous study on mycotoxin
259 bioaccessibility (González-Arias *et al*., 2013).
260
261 Regarding formation of OTα metabolite, generally low amounts were detected in our *in vitro*
262 digestion system. In the gastric chyme, OTα was detected in a range representing 5.1-19.1% of
263 the initial OTA, and in the small intestine juice 5.1% of the metabolite was detected only in the
264 digestion of the wine spiked at 4 μg OTA/mL. In the dialysis fraction, OTα was detected but not

265 quantified because the signal was below LOQ. Our results are in agreement with Madhyasta *et*
266 *al*. (1992); the authors used an *in vitro* system based on the incubation of OTA with rat digesta,
267 and found that most OTA hydrolysis to OTα occurs in the presence of digesta from the large
268 intestine or ceacum, but little hydrolysis was observed in the stomach contents and digesta from
269 the small intestine, despite the fact that the pancreatic enzymes, including carboxypeptidase A,
270 are released into the small intestine. Additionally, the biotransformation of OTA into OTα is
271 probably higher *in vivo* or *in vitro* systems involving intestinal cellular systems in comparison to
272 our *in vitro* system, where scarcely any biotransformation was seen. In our results, as in the case
273 of other studies, several additional factors were not taken into account, e,g., the interaction of the
274 toxin with the intestinal microbiota in the large intestine (mainly anaerobic bacteria)
275 (Madhyastha *et al*., 1992), the effect of the toxin´s metabolism during the transport through the
276 intestinal barrier and liver degradation on the *in vivo* oral bioavailable OTA (Berger *et al*., 2003;
277 Schrickx *et al*., 2006).
278
279 **4. Conclusions**
280
281 The present study shows that OTA is mainly released from the wine matrix during gastric
282 digestion (providing high bioaccessibility at this point). However, most probably the *in vivo*
283 bioavailability of this toxin decreases due to a low bioaccessibility, and presumably low
284 bioavailability, at the small intestine.
285
286 *In vitro* models are only approximations of what may occur *in vivo* and the few studies carried
287 out to determine the bioaccessibility of OTA in different food matrices, naturally or artificially
288 contaminated, have given inconsistent results. Most *in vitro* bioaccessibility assays for
289 mycotoxins have not been carried out for more than two hours of gastric digestion and few
290 matrices have been used under fasting or fed conditions. Further studies should be conducted to
291 determine the effect that OTA contamination level, type of contamination (natural *vs* spiked),
292 and food matrices exerts on bioaccessibility. Additional data are required to consider these
293 results representative of what happens *in vivo* conditions.
294
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