1 **Determination of ochratoxin A in tissues of wild boar (*Sus scrofa L.*) by enzymatic**

2 **digestion (ED) coupled to high-performance liquid chromatography with a**

3 **fluorescence detector (HPLC-FLD)**

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15

16   **Abstract**

17   Ochratoxin A (OTA) is a secondary toxic metabolite synthesized by *Aspergillus* or

18   *Penicillium* species, which can contaminate various crops. The International Agency for

19   Research on Cancer (IARC) classified OTA as a group 2B possible human carcinogen.

20   The aim of the present study was to assess OTA concentrations in tissues of wild boar

21   (*Sus scrofa L.*) from Tuscany (Italy). Over a period of 2 years, samples of muscle, liver

22   and kidney from 48 wild boars were collected and concentrations of OTA were

23   determined by enzymatic digestion (ED) coupled to high-performance liquid

24   chromatography with a fluorescence detector (HPLC-FLD). The highest concentrations

25   of OTA were found in the kidneys of the 48 wild boars analyzed. No difference in

26   concentrations were found based on years of collection and sex while a significantly

27   higher OTA concentration was found in the kidney of the young wild boars respect to

28   the adult one. Monitoring the quality of meat destined for transformation is a priority in

29   order to decrease the possibility of toxin carry-over to humans. The present study

30   showed that contamination of wild boar meat products by OTA represents a potential

31   emerging source of OTA.

32

33   **Keywords**: Ochratoxin A, wild boar, muscle, kidney, liver, HPLC

34

Introduction

Ochratoxin A (OTA) is a secondary toxic metabolite of various *Penicillium* and *Aspergillus* fungi, which is widely distributed in various food commodities, including cereals, coffee, beer, wine and spices (Malir et al., 2016). OTA has carcinogenic, nephrotoxic, teratogenic, immunotoxic and possibly neurotoxic properties (EFSA, 2006). International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogen (Group 2B) (IARC, 1993). Long-term exposure to OTA in humans has been implicated in Balkan endemic nephropathy (BEN) and is associated with urinary tract tumors because of the high OTA levels detected in food samples and in biological fluids from affected patients (Fuchs and Peraica, 2005; Pfohl-Leszkowicz et al., 2002). As cereals are widely used in animal feed, animals are potentially exposed to OTA through the consumption of contaminated feed, which can lead to the accumulation of this mycotoxin in meat and meat products (EFSA, 2004). Ranging OTA levels of 0.1-1 µg/kg have been detected in foodstuffs of animal origin (pork and chicken meat, dry-cured ham) (European Union, 2002; EFSA, 2004; Ostry et al., 2015). The European Union has established regulatory levels of OTA in cereals and in a wide variety of other food, but has not established maximum OTA levels in meat-based products (EC, 2006a). However, some countries have set maximum levels of OTA in meat or animal products, such as Denmark (pig kidney 10 µg/kg, pig blood 25 µg/ml) and Romania (pig kidney, liver, and meat 5 µg/kg) (EFSA, 2004). Also, Italy through the Italian Ministry of Health Circular No 10, dated 9 June 1999 (Italian Ministry of Health, 1999), establishes, as a guideline, a maximum level (ML) for pork meat and meat products of 1 µg/kg OTA.

Wild ungulate populations are increasing in Italy, especially in Tuscany where wild ungulate meat derives mainly from wild boar. It is estimated that these animals are

around 300,000 and the consumption rises to significant levels until 4 kg per capita/year

(Ramanzin et al., 2010). The significant increase in the wild boar population has

resulted in an increased prevalence of wild boar meat and ready-made products in the

food industry. Furthermore, an increasing interest in meat from animals kept in

conditions as close as possible to natural one has been developed in recent years,

because of special sensory properties desired by consumers (Vergara et al., 2003;

Soriano et al., 2006). For these reasons, there is an increasing awareness of the

importance of implementing good practices to ensure safety of meat obtained from

game.

The available knowledge on xenobiotics contamination of meats from wild ungulates

reported data regarding heavy metals, organochlorine pesticide and polychlorinated

biphenyl contamination (Bilandžić et al., 2009; Chiari et al., 2015; Mulero et al., 2016;

Naccari et al., 2004), whereas few data are available on mycotoxins contamination in

these species (Bozzo et al., 2012; Grajewski et al., 2012). In general compared to farm

animals, these species showed higher levels of contamination by xenobiotics such as

heavy metals and organochlorine compounds, which can constitute a greater

toxicological threat (Larsen et al., 2010; Rudy, 2010).

The aim of the present study was to determine OTA concentrations in muscle, kidney

and liver of wild boars hunted in Tuscany between 2014 and 2015 by using an

enzymatic digestion (ED) method coupled to high-performance liquid chromatography

with a fluorescence detector (HPLC-FLD).

Materials and methods

Samples

The research was conducted with the participation of the Migliarino, San Rossore, Massaciuccoli (MSRM) Natural Park staff. The MSRM is a regional park stretching along the Tyrrhenian coast on an area of about 240 sq km, located in Pisa and Lucca provinces. Inside the park there are several species of wild animals specially ungulates as wild boar. This specie is the most numerous and represents environmental interest. The management of the animals includes a series of reliefs and catches during the seasons. Periodically biologists estimated the population across the transect method to verify the real number of the individuals. The management of these ungulates is essential to sustain the population avoiding that it can reduce the natural resources available for the future.

For the present study, sampling of animal was carried out during periods of planned felling performed by the MSRM Park staff; the killings were carried out according to a planning in the different areas where the animals appeared to be in surplus. The culled wild boars were recovered, transported to a slaughterhouse and eviscerated in accordance with the EU regulations (EC 2004a; EC 2004b). For each animal was compiled a data sheet where it was reported the day of killing, abatement area, sex and age-class choice of three periods (0-4 months; 4-12 months, > 12 months) depending on the pigmentation of the coat of the animal killed (light brown coat; reddish coat; dark brown cloak). During the cutting operations the carcass was weighed and portions of liver, kidney, fat and muscle were taken from each one. Muscle samples were portions of the inner muscle of the back thigh of the animal. The samples were stored at a temperature of -18 ° C pending the analytical determinations.

Forty eight wild boars (male n = 25, female n= 23) culled from 2014 to 2015 have been slaughtered and the carcass weight were determined (from a min. of 13.8 kg and a max.

109   of 72.0 kg).

110   We had selected these provinces due to the large number of wild boars being shot there,

111   in order to decrease damage to crops caused by wild boars.

112   *Reagents*

113   OTA (from *Aspergillus ochraceus*) (M 403.8) reference standard was purchased from

114   Sigma (Milan, Italy). The OTA standard was dissolved in a toluene-acetic acid mixture

115   (99:1 %, v/v) to give a stock solution of 200 µg/ml, which was stored at -20 °C until

116   use. Working solutions (0.01, 0.02, 0.1, 0.2, 0.5 and 1 µg/ml) were prepared by diluting

117   the stock solution with the mobile phase consisting of a methanol-sodium phosphate

118   buffer (pH 7.5) 50:50 % v/v. HPLC-grade water, methanol, ethylacetate and acetonitrile

119   were purchased from VWR (Milan, Italy). The pancreatin enzyme (from porcine

120   pancreas) was purchased from Sigma (code P1750, Milan, Italy), and was stored at – 20

121    $^{\circ}$C until use.

122   *Chromathographic method*

123   The chromatographic system consisted of a Jasco 880 pump and a Jasco 821

124   fluorescence detector (Jasco, Tokyo, Japan). JascoBorwin software was used for data

125   processing. The excitation wavelength (λex) and emission wavelength (λem) were set at

126   380 and 420 nm, respectively. The reversed-phase column was a HAISIL HL, C_{18}, 5

127   µm, 150 mm x 4.6 mm (Higgins Analytical, USA). The column was kept at room

128   temperature. The HPLC was operated with a mobile phase system consisting of a

129   methanol-phosphate buffer solution pH 7.5 (0.03 M Na_2HPO_4, 0.007M NaH_2PO_4) 50/50

130   % v/v at flow rate of 1 ml/min.

131   *Enzymatic digestion method*

132   Samples were extracted using the method of Luci et al. (2016). Five grams of muscle,

133   liver, or kidney sample aliquot were homogenized with 5 ml of a phosphate buffer

134   (sodium phosphate monobasic dihydrate 0.2 M and sodium phosphate dibasic 0.2 M

135 20:80 % v/v pH 7.5) using an Ultra Turrax T25 homogenizer for a few minutes. A 2.5 g

136 aliquot of the homogenate was transferred into a tube and incubated for 1 hour at 37°C

137 with 5 ml of 1% pancreatin solution of in a phosphate buffer (sodium phosphate

138 monobasic dihydrate 0.2 M and sodium phosphate dibasic 0.2 M 20:80 % v/v pH 7.55).

139 The incubation was performed in a rotatory shaker, after which step samples were

140 acidified with 85% H_3PO_4 until pH 2-3. These samples were then extracted with the

141 same volume of ethylacetate, vortexed for 1 min, and centrifuged for 10 min at 3000

142 rpm. The organic phase was evaporated to dryness under nitrogen stream, reconstituted

143 in 1000 μl mobile phase, and a 100 μl aliquot was injected into HPLC.

144 *Method validation*

145 The HPLC-FLD method was validated according to (EC, 2002, EC 2006b) by

146 evaluating: specificity, recovery, trueness, decision limit (CCα), detection capability

147 (CCβ) of the method, linearity, LOD and LOQ, repeatability and reproducibility. A ML

148 of 1 μg/kg (1 ppb) OTA in pork meat and derived products was established by the

149 Italian Ministry of Health in 1999 (Italian Ministry of Health, 1999). The validation

150 procedure was performed taking into account the ML of 1 μg/kg OTA.

151 The linearity was evaluated by spiking muscle, liver and kidney samples with OTA at

152 0.05, 0.1, 0.5, 1, 2.5 and 5 μg/kg and analysing them using the extraction and HPLC-

153 FLD method. The experiment was repeated three times. The repeatability was tested by

154 analysing muscle, liver and kidney samples spiked with OTA at the levels of 0.1 μg/kg,

155 1 μg/kg, and 5 μg/kg. All samples were measured in triplicate on the same day. For the

156 within-laboratory reproducibility test, each of the fortification levels was tested in

157 triplicate over a period of five days. The results of these experiments were also used for

158 the determination of the recovery. No certified reference material was available for the

159 trueness assessment of OTA analysis in wild boar tissues samples. Repeatability and

160 reproducibility data corrected with the mean recovery were used for trueness

161   determination; trueness (%) was calculated as the mean (recovery corrected)

162   concentration of added known amount x 100 /added amount. The LOD and LOQ were

163   determined by the signal-to-noise approach, defined at levels resulting in signal-to-noise

164   ratios of 3 and 10, respectively. The analytical response and the chromatographic noise

165   were measured from the chromatogram of a blank sample extract (1 ml) to which an

166   OTA solution was added. The decision limit was estimated by spiking 10 muscle, liver

167   and kidney samples at the current limit taken as the reference value (1 µg/kg). The

168   concentration at this limit plus 1.64 times the corresponding standard deviation equals

169   the decision limit ($\alpha = 5\%$). Decision capability was estimated by spiking 10 muscle,

170   liver and kidney samples at the corresponding CCα level. The value of the decision

171   limit plus 1.64 times the corresponding standard deviation equals the decision capability

172   ($\beta = 5\%$).

173   *Statistical analysis*

174   Statistical analysis was performed with GraphPad Prism (v. 6) software (La Jolla, CA,

175   USA). All data were tested for normality by means of the Kolmogorov-Smirnov test.

176   The data are reported as median and range. A value of $p < 0.05$ was considered

177   significant. Linear regression analysis and Spearman correlation coefficient analysis

178   were used to assess the correlation between OTA concentrations in muscle, liver,

179   kidney and wild boar body weight. Mann-Whitney test was used to compare OTA

180   tissues concentrations between female and male wild boars, between old and young

181   wild boars and between wild boars from the two different years of the study.

182

Results and discussion

Environmental contamination and food safety are a major public health concern worldwide and several studies showed that mycotoxins occurred very frequently in food (Alshannaq and Yu, 2017; Capcarova et al., 2016). ~~The increase in the wild boar population in Europe is correlated with an increase in the consumption of wild boar meat, which may involve a risk of xenobiotics carry-over to consumers.~~

The analytical method used in the present study has been showed to be easy and time-saving, accurate and can be applied at levels of OTA contamination considerably lower than the ML, which is indicated by Italian Ministry of Health Circular No 10 dated 9 June 1999 (Italian Ministry of Health, 1999). The extraction phase is a critical point of the analytical procedure, because is highly influenced by the matrix. The most common extraction procedures for the determination of OTA in animal tissues are performed by using chloroform, followed by a clean-up with immunoaffinity columns or liquid-liquid partitioning (Valenta, 1998; Curtui et al., 2001; Monaci et al., 2004). These techniques need a large amount of organic solvents, which are environmentally harmful.

The enzymatic digestion technique used in this study allowed to reduce the use of organic solvents in comparison to other methods and to avoid the use of immunoaffinity columns which are expensive. Furthermore, enzymatic digestion method is simple, easy to apply and shows very satisfactory performance criteria. Figure 1 showed chromatograms of muscle, liver and kidney samples naturally contaminated by OTA of one wild boar. Results of the validation study are reported in Table 1. The method has been validated according to EU criteria for the confirmatory methods for contaminants and has shown to be suitable for accurate quantitative determination of OTA in different tissues of wild boars.

The median and the range of OTA concentrations found in the muscle, liver and kidney of wild boars sampled in the two years analyzed are shown in Table 2. The highest

concentrations of OTA were found in the kidneys of the 48 wild boars analyzed. The

levels found in the liver were 4 (year 2014) and 2 (year 2015) fold lower than in the

kidneys. The lowest concentrations were found in muscle samples.

There weren't significant differences between OTA concentrations in muscle, liver and

kidney samples between the two years analyzed. No significantly differences were

found in OTA concentrations between female and male wild boars in the entire studied

period and also in single years (Table 2).

The present results are in agreement with previous study conducted in Italy and Poland

in wild boars (Bozzo et al., 2012; Grajewski et al., 2012). Swine are particularly

sensitive to OTA, kidneys showed the highest accumulation of the toxin, followed by

liver and muscle tissue. ~~finally the lowest accumulation is represented in adipose tissue~~.

The present results showed the same type of accumulation in wild boar tissues. ~~The year~~

~~of sampling of wild boar did not influence the OTA concentrations in muscle, liver and~~

~~kidneys samples as the sex of animals. The age of animals influenced the OTA~~

~~concentrations particularly in the kidney samples.~~

Spearman analysis revealed statistically significant correlation between muscle, liver

and kidney OTA concentrations (Table 3). Spearman analysis for the relationship

between body weight and OTA in kidneys showed a statistically significant negative

correlation. As body weight increased the OTA content decrease and animals with a

body weight > 50 kg can be considered to have only trace amounts of OTA in their

kidneys (Figure 2). Spearman tests revealed no significant correlation between body

weight and OTA concentrations in both muscle and liver (Figure 2). The OTA

concentrations in the kidneys were significantly higher in young than in adult wild

boars, while no significantly differences were found in OTA concentrations in muscle

and liver between young and adult wild boars (Table 4).

234 ~~Adult wild boar showed lower OTA concentrations in kidneys than the young.~~ These

235 results were also confirmed by correlation analysis between OTA concentrations in

236 kidney samples and body weight of wild boar. As previously reported by Grajewski et

237 al. (2012) we also found that animals weighing < 50 kg accumulated much OTA in their

238 kidneys. Adult wild boars migrate in search of food resulting in the ingestion of more

239 diversified (i.e. varied) feed and in a reduction of the time they spend in each feeding

240 area. However, hungry young pigs consume large quantities of feed at a time that makes

241 them subjects to a more rapid and intense mycotoxin exposure. These results are

242 different from studies of heavy metal contamination in wild boar tissues that showed a

243 positive linear relationship between OTA content and weight of animal (Larsen et al.,

244 2010, Rudy, 2010). This result could be explained by the different metabolism of OTA

245 respect to inorganic compounds such as lead.

246 In 16 of the tissue samples examined in this study (10 kidney, 6 liver), the

247 concentrations of OTA were higher than the ML (1 µg/kg) established by the Italian

248 Ministry of Health Circular No 10 dated 9 June 1999 (Italian Ministry of Health, 1999).

249 None of the muscle samples exceeded the limit. The correlation between the OTA

250 content in muscle and in kidneys was positive and high, indicating a positive

251 proportional increase of OTA between the two tissues. The higher level of OTA in the

252 kidney was probably connected to the high affinity of OTA for this tissue and to the low

253 rate of excretion. Its bioavailability in monogastric species is high and it accumulates in

254 the blood and edible organs, especially in the kidneys (Ringot et al., 2006).

255 OTA can be carried over to wild-boar meat products and can also be present in the

256 muscle tissue. ~~Maximum levels of some mycotoxins have been legally set for several~~

257 ~~food products but until now such regulations do not apply to meat products.~~

258 ~~Nevertheless, a revision of the OTA limit in meat products is expected, according to the~~

259 ~~risk assessment updating (EC, 2005).~~

Furthermore, traditionally in several Italian regions, wild boar meats are used to produce niche products, especially coppa and salami. These derived products throughout the ripening time could be contaminated with higher OTA concentration because of water loss of in the drying stage. For example, Markov et al (2013) reported OTA contamination in wild boar fermented sausage, range of concentrations 2.70-3.07 µg/kg, suggesting the enrichment of OTA levels in ripened meat products. For these reasons dried wild boar meat may contribute to overall OTA intake by carry-over effects into processed meats. Monitoring the quality of meat destined for transformation is a priority in order to decrease the possibility of toxin carry-over to humans. Several recent studies indicated OTA occurrence in pig tissues from sub-chronically treated pigs (Persi et al., 2014; Pleadin et al., 2016). OTA was also found in pig tissues naturally contaminated (Matrella et al., 2006). OTA tissues levels reported in the present study are close to the levels of naturally contaminated pigs tissues and lower than sub-chronically treated pigs. Despite of the fact that OTA levels established in game meat within this study results appear not to be hazardous for human health if entering the human body via game meat consumption route, and despite of the fact that game meat is generally not consumed in large amount, MLs for various types of meat and meat derived products should be defined.

Conclusion

The present study confirms that contamination of meat products by OTA represents a potential emerging source of OTA. Because wild ungulates meat is being consumed in increasing quantities and no EU legislation on minimum risk levels in their meat exists, consumers may be at risk from the effects of potentially highly toxic contaminants such as OTA. Therefore, there is a clear need to establish a specific regulatory framework for contaminants in wild ungulates meat.

290

Conflict of interest None.

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406 **Figures legend**

407 Figure 1. HPLC-FLD chromatograms of muscle naturally contaminated at 0.74 µg/kg
408 A), liver naturally contaminated at 1.61 µg/kg B) and kidney naturally contaminated at
409 1.90 µg/kg C) samples of one wild boar.
410 Figure 2. Correlation between body weight and OTA concentrations in muscle (r = -
411 0.074, p = 0.628) liver (r = - 0.189, p = 0.214) and kidney (r = - 0.338, p = 0.023)
412 samples.
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414

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Table 1. Validation parameters of ED method coupled with HPLC-FLD according to (EC, 2002, EC 2006b); (LOD = limit of detection, LOQ = limit of quantification, r2 = coefficient of correlation, SD = standard deviation, RSD = relative standard deviation).

Parameters		Muscle	Liver	Kidney
LOD (µg/kg)		0.001	0.001	0.001
LOQ (µg/kg)		0.002	0.002	0.002
r²		0.997	0.999	0.998
Repeatability				
0.1	Mean concentration ± SD	0.088 ± 0.003	0.095 ± 0.002	0.093 ± 0.004
	RSD (%)	3.01	2.11	4.93
1.0	Mean concentration ± SD	0.92 ±0.03	0.92 ± 0.02	0.94 ± 0.04
	RSD (%)	3.80	2.75	4.44
5.0	Mean concentration ± SD	4.92 ± 0.05	4.98± 0.02	4.96 ± 0.01
	RSD (%)	1.00	0.40	0.12
Reproducibility				
0.1	Mean concentration ± SD	0.090 ± 0.003	0.094 ± 0.003	0.093 ± 0.003
	RSD (%)	3.95	2.82	3.63
1.0	Mean concentration ± SD	0.94 ± 0.03	0.93 ± 0.02	0.94± 0.04
	RSD (%)	3.50	2.68	3.92
5.0	Mean concentration ± SD	4.93 ± 0.06	4.97 ± 0.03	4.92 ± 0.10
	RSD (%)	0.93	1.11	0.81
Recovery %				
0.1		86.11 ± 3.54	94.50 ± 2.67	93.50 ± 3.39
1.0		94.00 ± 3.29	93.33 ± 2.50	93.68 ± 3.67
5.0		98.70 ± 0.92	98.63 ± 1.10	98.67 ± 0.80
CCα		1.031	1.069	1.059
CCβ		1.06	1.100	1.098

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421 **Table 2. Median and range of OTA concentrations (μg/kg) in the two years analyzed; LOD = limit**
422 **of detection.**

	2014		
	Muscle	**Liver**	**Kidney**
Female (n = 12)	0.10 (<LOD-0.77)	0.15 (0.06-1.93)	1.10 (0.24-1.90)
Male (n = 14)	0.08 (<LOD-0.77)	0.15 (0.04-1.76)	0.63 (0.19-3.23)
Total (n = 26)	0.08 (<LOD-0.77)	0.15 (0.04-1.93)	0.68 (0.19-3.23)
	2015		
	Muscle	**Liver**	**Kidney**
Female (n = 11)	0.18 (0.03-0.38)	0.23 (0.05-1.31)	0.45 (0.07-1.72)
Male (n = 11)	0.12 (0.06-0.50)	0.20 (0.02-0.91)	0.25 (0.09-1.15)
Total (n = 22)	0.13 (0.03-0.50)	0.23 (0.02-1.31)	0.34 (0.07-1.72)

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426 **Table 3. Spearman correlation analysis between muscle, liver and kidney OTA concentrations.**

	p	R^2	r
Muscle vs Liver	<0.0001	0.33	0.64
Muscle vs Kidney	<0.0001	0.70	0.62
Kidney vs Liver	<0.0001	0.46	0.57

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Table 4. Median and range of OTA concentrations (µg/kg) in young and adult wild boars; LOD = limit of detection; different letters indicate significant difference (p<0.05) between samples of young and adult using Mann-Whitney test.

	Young (n = 9)	Adult (n = 39)
Muscle	0.10 (<LOD-0.77)[a]	0.08 (<LOD-0.77)[a]
Liver	0.15 (0.06-1.93)[a]	0.15 (0.04-1.76)[a]
Kidney	1.10 (0.24-1.90)[a]	0.63 (0.19-3.23)[b]

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