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1 **Ochratoxin A in artisan salami produced in Veneto (Italy)**

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10 **ABSTRACT**

11 Fifty samples of artisan salami purchased in Veneto (Italy) were analysed for the determination of
12 ochratoxin A (OTA). The analytical method, based on a sample preparation procedure with
13 immunoaffinity columns (IACs), together with analysis by high-performance liquid chromatography
14 with fluorescence detection (HPLC-FD), has guaranteed a high rate of recovery (about 97%), limit
15 of detection (LOD) and limit of quantification (LOQ), respectively, of 0.06 $\mu\text{g kg}^{-1}$ and 0.20 $\mu\text{g kg}^{-1}$.
16 OTA was detected in five samples, but only one exceeded the guideline value (1 $\mu\text{g kg}^{-1}$) established
17 by the Italian Ministry of Health for pork meat and derived products. The results would seem to
18 suggest that salami made with the traditional, non-industrial production method can be considered
19 safe as regards contamination by OTA. However, the very high concentration observed in one sample
20 proves that a high OTA contamination is also possible in this type of product. Thus, the controls of
21 mycotoxin contamination must consider also salami.

22

23 **KEYWORDS:** Mycotoxins, ochratoxin A, salami, HPLC, immunoaffinity, fluorescence detection

24

25 **Introduction**

26 Salami (Salame in Italian) is a typical product of the Italian sausage tradition. Salami is defined as a
27 charcuterie product, consisting of meat obtained from striated muscles belonging to pig carcass with
28 the addition of sea salt and eventually meat of other animal species, minced and combined in varying
29 proportions with pork fat, and packed into natural or artificial casings (Ministero delle Attività
30 Produttive 2005). The meat is allowed to ferment before being packed into casings and then hung up

31 to cure. The curing process activates bacteria, which makes the ground meat an inhospitable
32 environment for dangerous bacteria that can cause meat to spoil. In this way, the product is safe to
33 eat, without refrigeration, for several years. The long shelf life is one of the reasons why such products
34 were so popular before the development of the modern refrigeration systems. Salami is a generic term
35 indicating a large group of meat products. Each Italian region produces specific types of salami that
36 can differ on the basis of various criteria: the origin of the meat, the methods of chopping and
37 preparation of the lean and the fatty parts (which can be chopped fine, coarse or into little cubes), the
38 ratio between the different parts, the salting and the addition of different spices, the type of casing,
39 the size of the final product, the development of moulds on the casing and the seasoning methods.
40 The production of salami is often linked with local customs and traditions and represents a real
41 cultural heritage; several consortiums have been established for the protection of these products and
42 to obtain the recognition of them as Protected Designation of Origin (PDO) or Protected Geographical
43 Indication (PGI) product.

44 Ochratoxins are mycotoxins produced by various *Aspergillus* and *Penicillium* spp., particularly
45 *Penicillium verrucosum* and *Aspergillus ochraceus* (Kuiper-Goodman & Scott 1989). They are
46 widely spread in the world, reaching a high level of contamination, especially in some countries of
47 Northern Europe and South America (Devegowda et al. 1998). These ubiquitous saprophytic moulds
48 can contaminate grains, feed and food; they develop during the crop growth, but also during the
49 storage phase. The ochratoxin-producing fungi, for the growth in cereals, require a minimum moisture
50 content of 17–18% and temperatures of 8–37 °C. The genus *Aspergillus*, more commonly associated
51 with warmer climates, prefers food products with high fat and protein content like sausages, while
52 *Penicillium* is the typical storage mould and it grows in cool temperate climates, preferring cereals
53 rich in carbohydrates such as barley and wheat (Sweeney & Dobson 1998; Magan & Aldred 2005;
54 Cicoňova et al. 2010). There are three ochratoxin compounds, ochratoxin A (OTA), ochratoxin B
55 (OTB) and ochratoxin C (OTC); OTA is the most common one and contaminates various types of
56 food of plant origin as well as some animal products such as liver and kidney. In terms of toxicity,
57 OTA and OTC are the most dangerous, while OTB has toxicity values 10 times lower than that of
58 OTA (Marquardt & Frohlich 1992). OTA is a very stable and persistent molecule and it is responsible
59 for many carcinogenic, nephrotoxic, immunotoxic, teratogenic, neurotoxic and genotoxic effects on
60 laboratory and farm animals. OTA is classified as possibly carcinogenic to humans (IARC 1993).

61 Regarding the main sources of human exposure to OTA, a survey carried out at the European level
62 shows that cereals are the primary source of contamination (50%) by OTA. Other matrices affected
63 by contamination are wine (13%), coffee (10%), spices (8%), beer (5%), cocoa (4%), dried fruit (3%)
64 and meat (1%) (European Commission 2002; Jørgensen 2005). The Commission of the European

Communities fixed maximum levels in several foodstuffs (European Commission 2006a). It is important to remember that OTA may be present in animal feed, but meat, milk and eggs from animals fed with contaminated feeds have so far been considered a negligible source for human exposure. However, higher concentrations of OTA may be present in certain local specialties such as blood puddings and sausages prepared with pig blood serum (EFSA 2006). Between farm animals, the pig is known to be particularly sensitive to OTA, which can be found in decreasing concentration in kidneys, then liver and muscles (Curtui et al. 2001; Malagutti et al. 2005) and fat (Krogh et al. 1976; Madsen et al. 1982). The Commission of European Communities fixed, in Recommendation 2006/576/EC, a guidance value for OTA of 0.05 mg/kg in complementary and complete feeding stuff for pigs (European Commission 2006b), but didn't set maximum OTA levels in meat-based products. However, in Italy, since 1999 the Ministry of Health has established a guideline OTA value of 1 $\mu\text{g kg}^{-1}$ in pork meat and derived products (Ministero della Sanità 1999).

The aim of this study was to carry out a monitoring action to assess the presence of OTA in artisan salami sampled in Veneto (Italy), using a suitable analytical method for the evaluation of OTA in highly heterogeneous meat products such as salami. In the Veneto region, there is a long tradition in the production of handmade salami from small family-run farms and holiday farms. Since these small farms have no self-control systems like the major meat product factories, it becomes interesting to assess whether OTA, in these small productions, constitutes a real risk to human health.

Material and methods

Salami samples

From September to October 2013, 50 different salamis were purchased in a random manner from family-run farms, holiday farms and mountain huts ("malghe") located in Veneto, in the provinces of Vicenza and Treviso. In particular, the samples were 14 fresh salamis, five aged salamis and 31 sopressa (typical Venetian salami). After purchase, the samples were registered and catalogued in the laboratory notebook. From each salami, three 100 grams aliquots (cross sections) were sampled in different positions and collected after removing the casing; the aliquots were minced together using a mini grinder (Illico, Moulinex, France) and stored at $-20\text{ }^{\circ}\text{C}$ until analysis.

Solvents and reagents

The chemicals and solvents used for the extraction of OTA from salami samples (ethyl acetate, phosphoric acid, sodium bicarbonate), as well as the chemicals used to prepare PBS buffer (sodium chloride, disodium hydrogen phosphate anhydrous, potassium phosphate monobasic, potassium chloride) were ACS grade (Carlo Erba Reagents, Cornaredo, MI, Italy). The solvents used in the post-

97 extraction immunoaffinity clean-up (water, methyl alcohol), and all solvents used for HPLC analysis
98 (water, acetonitrile, isopropyl alcohol, acetic acid), were HPLC grade (Mallinckrodt Baker B.V.,
99 Deventer, The Netherlands). OTA standard was purchased from Sigma-Aldrich Co. (St Louis, MO,
100 USA). Immunoaffinity columns (IACs) used for sample purification (OchraTest™ WB) were
101 purchased from Vicam® (Milford, MA, USA).

102 **Chromatographic apparatus**

103 The HPLC system consisted of a System Gold Programmable Solvent Module 126 pump (Beckman
104 Coulter, Brea, CA, USA) equipped with an HT 800 L autosampler (HTA, Brescia, Italy) fitted with
105 a 20 µl loop and a 821 FP fluorescence detector (Jasco, Tokyo, Japan); fluorescence excitation and
106 emission wavelengths were 340 and 460 nm respectively. The system was computer-controlled by a
107 Beckman Coulter 32 Karat Software. The HPLC column was a C18 Onyx Monolithic column 100
108 mm x 4.6 mm (Phenomenex, Torrance, CA, USA) coupled in sequence to a C18 Chromolith
109 Performance RP-18e column 100 mm x 4.6 mm (Merck, Darmstadt, Germany). Chromatographic
110 separation was achieved in gradient conditions and at room temperature. The mobile phase consisted
111 of water-acetonitrile-isopropyl alcohol-acetic acid 1% mixtures in various ratios. Mobile phase A:
112 water/acetonitrile/isopropyl alcohol/acetic acid 1% (79:7:7:7 v/v) and mobile phase B: acetonitrile.
113 Gradient: from 80% A and 20% B to 58% A and 42% B in 11 min with linear increase. Flow rate: 1
114 ml/min. The injection volume was 20 µl.

115 **Sample extraction**

116 The extraction of OTA from salami was carried out following the methods described by Monaci et
117 al. (2004), (2005) and Bozzo et al. (2012) with some modifications. A 2.5 g aliquot of minced sample
118 was acidified with 1.5 ml of 1 M phosphoric acid and homogenised in 5 ml of ethyl acetate for 3 min
119 using an Ultra-Turrax homogeniser. The sample was centrifuged for 3 min (1900 xg) and the upper
120 organic phase was transferred into a 15 ml conical centrifuge tube. The pellet was then extracted a
121 second time in the same manner, and after centrifugation, the second organic phase was removed and
122 combined with the first one. The combined extract, after being frozen (−20 °C) for 3–4 hours, was
123 centrifuged for 10 min (1900 xg) in order to separate emulsified and suspended components, which
124 were removed. An 8 ml aliquot of the extract (equivalent to 2 g sample) was then exactly measured
125 into a 10 ml graduated glass centrifuge tube and reduced to 2 ml with a Univapo rotational vacuum
126 concentrator (Uniequip, Martinsried/Munich, Germany). The organic extract was back-extracted by
127 adding 2 ml of 0.5 M sodium bicarbonate solution: the tube containing the two solvents was
128 thoroughly mixed for 30 min on a rotating shaker, frozen (−20 °C) for one hour and centrifuged for

129 10 min (1900 xg). Finally, the organic phase was removed and the bicarbonate extract was subjected
130 to clean-up.

131 **Sample clean-up**

132 An aliquot of 1.5 ml of bicarbonate extract was diluted with 3.5 ml of PBS buffer and loaded onto an
133 OchraTest™ WB IAC. The column was then washed with 10 ml of PBS buffer and 10 ml of water;
134 finally, OTA was eluted with 1.5 ml of methanol. The eluate was reduced to dryness by means of
135 Univapo and the residue redissolved in 150 µl of methanol, diluted with 150 µl of water, and after
136 vortexing, injected into HPLC.

137 **Quantification**

138 The parameters considered for the validation of the analytical method for the HPLC-FD detection of
139 OTA in salami were linearity and range, specificity, accuracy and precision, limit of detection (LOD),
140 limit of quantification (LOQ) and recovery. The evaluation of linearity and range was performed by
141 analysing five calibration standards of OTA in the range 0.2–2 µg kg⁻¹, prepared from blank samples
142 spiked directly with OTA standard solutions. Specificity, related to the absence of interfering
143 substances under the experimental conditions, was determined by calculating the mean values (±
144 standard deviation) of the retention time of OTA present in spiked samples and in unknown samples.
145 Accuracy and precision were evaluated via the analysis of spiked samples, using nine determinations
146 over three concentration levels in the range 0.2–2 µg kg⁻¹ (three concentrations/three replicates each).
147 Accuracy was expressed as the deviation of the mean from the true value, and precision as the relative
148 standard deviation (RSD) of the replicate measurements. The LOQ of the method was the lowest
149 concentration of the calibration curves of OTA. The LOD was established on the basis of a signal-to-
150 noise ratio of 3 at OTA retention time. The recovery was obtained by analysing pre-spiked extracted
151 samples in the range 0.2–2 µg kg⁻¹ compared with the detector response obtained for pure OTA
152 standard solutions at the same concentration levels. For its evaluation, at least three concentration
153 levels/three replicates each were used. For internal quality control purposes the unknown samples
154 processed the same day were always accompanied by one or more spiked blank samples. This study
155 was performed according to ISO 9001 (2008) requirements. Also, a z-score of -1.40 for OTA in
156 durum wheat at a level of 12.45 µg kg⁻¹ was obtained in a proficiency test (Bacer 2012).

157 **Results and discussion**

158 **Assay validation**

159 In the considered concentration range ($0.2\text{--}2\ \mu\text{g kg}^{-1}$), the calibration curves of OTA were linear
160 ($R^2 > 0.999$). The specificity of the method was acceptable. No interfering peaks were observed in
161 the spiked samples and no significant peaks were found within the retention time window of OTA in
162 the non-contaminated samples. Retention time for OTA was 14.09 ± 0.09 minutes. This value was
163 stable (RSD = 0.64% over 4 days). The run time was 16 minutes. Figure 1 shows the chromatograms
164 of a blank sample (A) and a naturally contaminated sample at $0.44\ \mu\text{g kg}^{-1}$ (B). Three calibration
165 standards at concentrations of 0.4, 1.0 and $1.5\ \mu\text{g kg}^{-1}$ were prepared and analysed at three different
166 days. The RSD for each concentration assayed never exceeded 3%. LOD and LOQ of the method
167 were $0.06\ \mu\text{g kg}^{-1}$ and $0.20\ \mu\text{g kg}^{-1}$, respectively, and were far below the guideline value of $1\ \mu\text{g kg}^{-1}$
168 established by the Italian Ministry of Health. Recovery rates were checked in three concentrations
169 and the overall average was 97.4%. For each concentration, three measurements were performed and
170 the RSDs ranged from 2.7 to 4.7% (Table 1). Since salami is a complex matrix and a non-
171 homogeneous material, it was critical to ensure the representativeness of the test sample that was
172 obtained by homogenisation of three sample aliquots taken at different points of each salami. The
173 clean-up method using IACs is fast, takes less than 15 min, and this made it possible to obtain
174 analytical samples without interfering substances in the chromatogram. Moreover, the gradient
175 developed for conducting HPLC analysis and the use of monolithic columns allowed good analytical
176 results.

177 **OTA in salami samples**

178 In this study 50 salami samples were analysed and five of them (10%) were positive for OTA (Table
179 2). Two of these positive samples showed very low OTA concentrations, just above the LOD. Another
180 two positive samples showed concentrations slightly above the LOQ. Only in one sample the OTA
181 concentration exceeded the guidance value of $1\ \mu\text{g kg}^{-1}$ as established by the Italian Ministry of
182 Health (Ministero della Sanità 1999). Given the very high concentration of this sample, for its
183 quantification, it has been properly diluted before HPLC analysis. Examining the data of this study
184 on the occurrence of OTA in salami, some considerations can be made. The percentage of positive
185 samples and the low concentration, below the guidance value of $1\ \mu\text{g kg}^{-1}$ for OTA in meat, found in
186 four out of five samples, suggests that artisan salami is a safe food regarding the presence of OTA.
187 On the other hand, the very high concentration observed in one sample (number sample-id 17) was a
188 value more than 100 times higher than the guidance value. This leads to the conclusion that a high
189 contamination by OTA is also possible in this type of product. The external appearance of this sample
190 was normal, without damages of casing; the shape was cylindrical, and regularly bound with
191 colourless twine. Also, the appearance on cutting was normal, and no existence of cracks was

underscored. Finally, the organoleptic characteristics (particular smell and flavour) were very good and no signs of spoilage were found. This salami deserves further comment about the possible cause of its contamination and about the causes of OTA contamination of salami in general. It is also conceivable that a similar amount of OTA might be the result of a combination of several adverse events. First, consideration should be given to the presence of OTA in muscle and fat of pigs used for the production of the contaminated sopressa, due to the assumption of contaminated feed. In this regard, the scientific literature reports several experiments in which tissues of various organs from pigs exposed to OTA-contaminated feed were analysed (Krogh et al. 1976; Raja et al. 2008). Other studies also considered final products prepared from pork meat. A recent article (Perši et al. 2014) reports data on OTA concentration in meat sausages produced from raw materials coming from pigs exposed to OTA-contaminated feed (300 µg OTA/kg feed) for 30 days. The results pointed out that a sub-chronic pig exposure leads to the accumulation of OTA in raw materials and consequently in meat products, whose level of contamination is directly dependent on OTA contents in raw materials used for their production.

An experiment conducted on growing pigs fed with OTA 25 µg kg⁻¹ contaminated feed for 119 days was reported by Malagutti et al. (2005). The pork meat was taken to make seasoned sausage and analyses were carried out to determine OTA in fresh sausages after 40 and 100 days of seasoning. Despite the OTA concentration in feed being below the limit established by current regulations (50 µg kg⁻¹), the final OTA content in meat was much higher than the Italian guideline value of 1 µg kg⁻¹. Seasoning and storage do not seem to have any effect on the reduction of OTA levels in meat products (Malagutti et al. 2005). However, if feed-associated intoxication was the only cause, the level of contamination detected in sample 17 is so high that it would be reasonable to assume that OTA concentrations in feed might have caused symptoms of ochratoxicosis in pigs. On the other hand, several authors report that indirect transmission of OTA from animals exposed to contaminated feed to pork products occurs rarely (Pietri et al. 2006; Iacumin et al. 2009). Some studies show that the extent of the contamination would be largely dependent on the environmental conditions typical of ageing and the characteristics of the production plant. If good manufacturing practices are not followed, or mistakes occur in the control of the relative humidity and temperature of the drying and ripening rooms, a significant growth of slime consisting of various moulds is often observed (Iacumin et al. 2009). A study carried out to assess the mycoflora in ham-manufacturing plants reports the constant presence of *Aspergilli* and *Penicillia*, potential mycotoxin producers, isolated from the air and the ham in all checked plants (Battilani et al. 2007). Another study reports that during the ripening process, a number of moulds colonise dry-cured sausages; temperature and salt content (which affects water activity, aw) predispose the surface to colonisation by *Penicillium* species, including

226 *Penicillium nordicum* and *Penicillium verrucosum*, which can lead to OTA contamination of the
227 sausages (Rodríguez et al. 2015). A study conducted at two Spanish dry-cured ham manufacturing
228 plants showed that the genus *Penicillium* represents the majority of the surface mycobiota of dry-
229 cured hams. It was also the predominant fungal genus in environmental samples, indicating that
230 fungal colonisation of dry-cured hams might be determined by fungi present in the environment of
231 ripening rooms. It also showed that a certain percentage of fungal strains (9.5%) belonging to different
232 *Penicillium* species was able to produce OTA (Alapont et al. 2014).

233 Additional possible sources of contamination are two ingredients normally used in the preparation of
234 salami: spices and red wine. The most utilised spices are garlic, anise, cinnamon, cloves, coriander,
235 fennel, nutmeg and pepper. Among these, OTA was reported as a natural contaminant in pepper and
236 nutmeg. However, the levels of mycotoxins in spices are generally low when compared with other
237 food products. Sometimes salami producers proceed with the addition of local wine, which further
238 characterises the harmony of different and pleasant flavours of typical Venetian salami. Wine brings
239 aromas and perfumes and plays an antiseptic and colour fixative action due to the sulphur dioxide
240 contained in it. On the other hand, wine is also considered, after cereals, one of the primary sources
241 of contamination by OTA. In fact, in a study at the European level also involving Italy, the percentage
242 of contaminated wine samples was very high (59.3%), and considering only data for Italy, the
243 percentage is even higher (80.6%). The levels of contamination ranged from 0.003 $\mu\text{g kg}^{-1}$ (Spain)
244 to 15.60 $\mu\text{g kg}^{-1}$ (Italy). Among the different types, red and sweet wines seem to be the most
245 contaminated (European Commission 2002). However, considering the low amount of spices and
246 wine in salami, it is unlikely that these ingredients cause high OTA contamination in the final product.

247 **Conclusion**

248 The analytical method, based on a sample preparation procedure with IACs, together with analysis
249 by HPLC-FD, is reliable for the determination of OTA in meat products like salami. This preliminary
250 study that considered a relatively low number of samples suggests that salami made with the
251 traditional, non-industrial production method, as regards OTA contamination, can be considered safe.
252 The very high concentration observed in one sample proves that a high contamination by OTA is also
253 possible in this type of product. Therefore, mycotoxin controls in food should also consider salami,
254 as well as other pig meat products, although these seem less at risk for this type of intoxication when
255 compared with cereals and other products of plant origin. It would be interesting to extend research
256 to salami producers in other areas.

257 **Disclosure statement**

258 No potential conflict of interest was reported by the authors.

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- 333 Figure 1. Chromatograms obtained on IAC purified extracts of (A) salami blank sample, and (B)
334 salami naturally contaminated with OTA at 0.44 µg kg⁻¹.

335

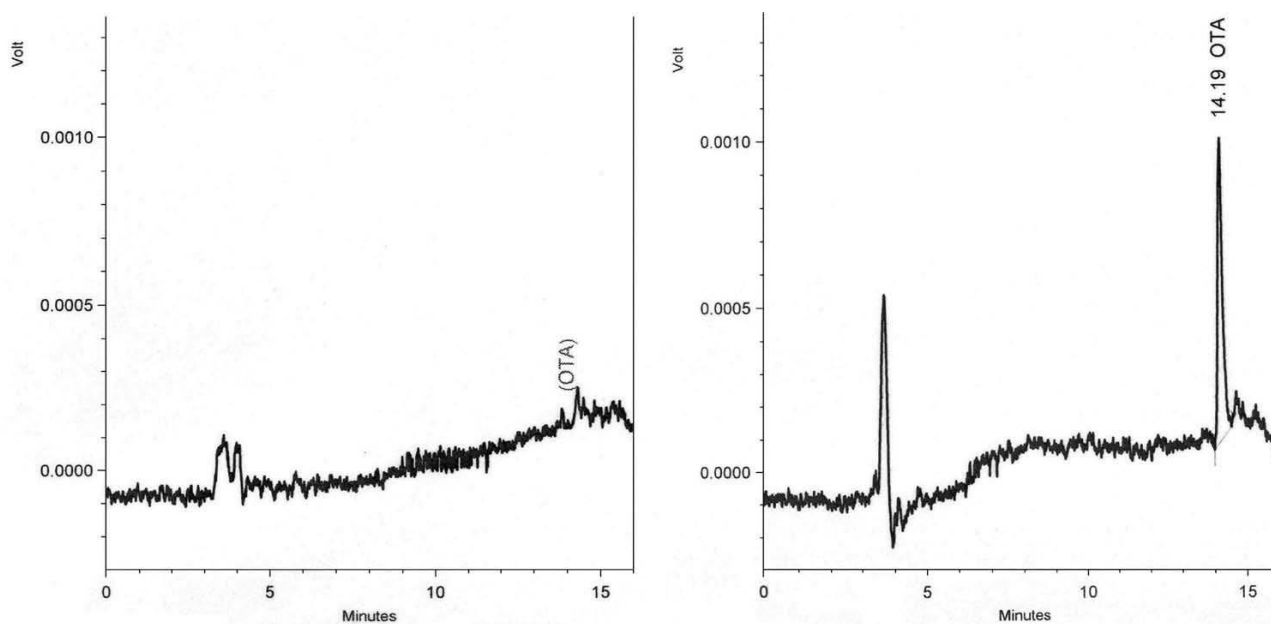


Table 1. Recovery and precision of the HPLC-FD method for OTA analysis in salami, as determined in triplicate.

Spike	Recovery	RSD
$\mu\text{g kg}^{-1}$	%	%
0.4	97.0	2.7
1.0	99.5	4.7
1.5	95.7	3.7

Table 2. Ochratoxin A concentrations in positive salami samples.

Sample id.	Type	$\mu\text{g kg}^{-1}$
9	Soprèssa	0.09
12	Soprèssa	0.44
17	Soprèssa	103.69
31	Soprèssa	0.06
32	Soprèssa	0.28