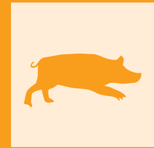
Natural occurrence of ochratoxin A in confined reared and grazing pigs derived products



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SUMMARY

Ochratoxin A (OTA) is a well-known nephrotoxic and immunotoxic mycotoxin occurring in several foods and feeds. OTA residues have been detected as a carryover in pigs and poultry meats. In this study samples of pigs fresh tissues (muscle, backfat) and processed meat products (seasoned muscle-coppa, seasoned backfat-lard, mortadella and salami) were obtained from pigs raised following two breeding systems: conventional production systems (indoor) and alternative pig rearing systems (free outdoor access). Pigs tissues and meat products samples were analysed for OTA content by means of an HPLC-FLD method. OTA was found in all pigs tissues and meat products at concentration lower than the Italian recommended maximum level of 1 µg/kg, only a sample of mortadella of indoor group showed a concentration very close to this limit. This study suggests that the contribution from pig fresh tissues to the total intake of the toxin is small if compared to other sources. However, the OTA occurrence in some derived products, such as mortadella, seems to be of some concern.

KEY WORDS

Ochratoxin A, pig, HPLC, meat, carry-over.

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced as secondary metabolite by *Aspergillus* and *Penicillium* spp. fungi during storage. OTA is nephrotoxic, immunosuppressive and teratogenic and has been classified by the International Agency for Research on Cancer as possible carcinogen Group 2B to humans[1,2]. Several studies suggest the involvement of OTA in the etiology of human Balkan Endemic Nephropathy (BEN) and of human chronic interstitial nephropathy in North Africa[3,4]. BEN has similarities with mycotoxic porcine nephropathy, which has been associated with pigs ingestion of OTA in Denmark in the 1960s and 1970s[5]. Public health concerns regarding OTA human exposure are justified on the basis of its demonstrated toxic effects, coupled with its ability to be carried through the food chain[6]. OTA can be detected in cereal products, coffee, wine, beer, cheese, and in food of animal origin. The latter can be contaminated either directly with toxigenic moulds or following the carry-over from naturally contaminated animal feed[7-10]. OTA concentrations in the range of 0.1-103.69 µg/kg have been detected in foodstuffs of animal origin (pork meat, dry-cured ham, sausage, salami)[10-12]. OTA-producing fungi can be recovered from the environment, the air of ripening plant rooms[13] and the surface of processed meat products[14,15]. The addition of flavouring materials, such as spices, may also be a supplementary source of mycotoxin contamination[16]. Non-ruminant mammals such as swine tend to accumulate OTA because of a rather long serum half-life of 72-120 hours.

Legislation regarding mycotoxins has been created to minimize the risk of food contamination by these compounds which are known to cause losses of quality in food products and adverse effects to human and animal health. Maximum levels of OTA in pigs meat or derived products have been set in some countries such as Denmark (pig kidney 10 µg/kg, pig blood 25 µg/ml), Estonia (pig liver 10 µg/kg), Romania (pig kidney, liver and meat 5 µg/kg) and Slovakia (meat 5 µg/kg, milk 5 µg/kg)[10]. Other countries such as Italy have developed guidelines for recommended maximum OTA levels (pig meat and derived products 1 µg/kg)[17]. On the contrary at the European level maximum limits of OTA in meat or other animal products has not been established. According to the EFSA[18] more extensive occurrence data on OTA in animal tissues and derived products are required to assess the significance of residue levels.

An increasing percentage of the population shows a preference for organically produced agricultural products due to the claimed absence of chemical contaminants within this mode of production. However, it is feared that products from organic agriculture could be more affected by mycotoxins than those from conventional agriculture, due to the lack of fungicide applications[19]. Very few data are available on the natural occurrence of OTA in products derived from pigs raised in organic conventional production systems (indoor) and alternative pig rearing systems (free outdoor access). Interest in outdoor pig production is growing in several countries because of better animal welfare and meat quality. The aim of the present study was to investigate the presence of OTA in tissues of pigs raised in an organic farm in

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different conditions (indoor and outdoor) during the fattening period; then, typical Italian seasoned pork products were prepared using the tissues and seasoned in a manufacturing plant. At the end of seasoning, analysis for OTA content was carried out on the seasoned products.

MATERIALS AND METHODS

In the present study, the pig production chain was analyzed from the breeding to the slaughterhouse up to the production of seasoned meat.

Animals

Ten Large White-Duroc crossbreed castrated male pigs (weight 30 ± 5 kg) were raised in an organic farm (Tuscany, Italy). During the fattening period 5 animals of the group were raised outdoors in a 5000 m^2 extension land characterized by chestnut trees, holm-oaks, heather, bramble shrubs (OTD group); the others 5 were raised indoors in a pen (IND group). All the animals were fed with the same diet. Flaked protein pea, flaked corn, flaked wheat, flaked barley was the composition of the same diet of the experimental groups (Table 1) according to requirements of National Research Council[20]. Animals had free access to water and the feed was distributed twice a day; OTD had the possibility to integrate their ration with woodland feed resources when available (chestnut, grass, roots).

Feed collection

A feed aliquot of flaked protein pea, flaked corn, flaked wheat and flaked barley for the analyses was collected at the beginning (T1) and end (T2) of the fattening period.

Tissues collection

When the pigs reached 140 kg of live weight were transferred to local slaughterhouse. During the slaughter samples of fresh *Longissimus lumborum* muscle and fresh backfat were collected from individuals of each group (OTD and IND).

Meat products collection

In a meat processing factory, meat derived products (seasoned muscle (coppa), seasoned backfat (lard), mortadella and salami), obtained from the same pigs sampled at the slaughterhouse, were collected. Coppa was obtained from muscle cut according to local processed technique, all fats were removed. After dry salting samples were washed, dried, trimmed and bagged in plastic wrap be aged in ventilated room for five months. Lard was obtained using about 5 kg of fresh backfat divided in small pieces of about 0.3 kg and placed into marble boxes. A layer of salt, minced garlic and mixture of herbs were added. Boxes were closed with a marble cover to limit light and air exposure during 15 days. Mortadella were obtained from a quantitative pork meat (in natural bowel) triturated and mixed with little backfat cubes. Samples were produced in batches of 50 kg each by mincing deboned shoulders (40%), porcine stomachs (25%), lard cubes from jowl (25%), fat emulsion (10%); mixing with salt, saccarose, caseinate, polyphosphates, sodium nitrite, pepper, garlic and stuffing in artificial casings. Cooking took place immediately in dry ovens at 85°C room temperature up to 72°C core temperature and was followed by cooling under tap water sprays and refrigeration in cold rooms at 4°C. Salami were produced by mincing and mixing deboned shoulders (30%), ham trimmings (40%), backfat (15%) and belly (15%). During mixing the following additives were added: salt, dried skimmed milk, saccarose, potassium nitrate, sodium nitrite. The mixes were stuffed in reconstructed casings and seasoned at 11-12°C and 75-85% RH up to 3 months from manufacture. The samples were obtained from the core of each product; all samples were vacuum-packaged and stored at -20°C until the analyses.

High-performance liquid chromatography method

OTA (from *Aspergillus ochraceus*) (M 403.8) reference standard was purchased from Sigma (Milan, Italy). The OTA standard was dissolved in a toluene-acetic acid mixture (99:1%, v/v) to give a stock solution of 200 µg/ml which was stored at -20°C until use. Working solutions were prepared by diluting the stock solution with the mobile phase consisting of methanol-sodium phosphate buffer (pH 7.5) 50:50% v/v. HPLC-grade water, methanol and acetonitrile were purchased from VWR (Milan, Italy). The immunoaffinity columns (IAC) OchraStar® were purchased from TecnaSrl (Trieste, Italy). The chromatographic system consisted of Jasco 880 pump and Jasco 821 fluorescence detector (Jasco, Tokyo, Japan). JascoBorwin software was used for data processing. The excitation wavelength (λex) and emission wavelength (λem) were set at 380 and 420 nm. The reversed-phase column was a Luna C18 ODS2, 3 µm (4.6 x 150 mm) (Phenomenex® Torrance, CA, USA). The column was kept at room temperature. The HPLC was operated with mobile phase system consisting of methanol-phosphate buffer solution pH 7.5 (0.03 M Na_2HPO_4, 0.007M NaH_2PO_4) 50/50% v/v at flow rate of 1 ml/min. The HPLC method was validated according to international rules[21]: selectivity, linearity, limits of determination (LOD) and quantification (LOQ), repeatability and reproducibility were determined. Calibration curves were based on the analysis of triplicate standards solution at 7 concentration levels in matrix. Meat samples spiked with OTA at 0.02, 0.05, 0.1, 0.25, 0.5, 1 and 2 µg/kg were analysed using extraction and HPLC method. Feed samples spiked with OTA at 0.2,

Table 1 - Ingredients and chemical composition of the diet.

		30-50 kg	50-90 kg	> 90 kg
Flaked protein pea	g/kg	450	350	300
Flaked corn	g/kg	200	300	350
Flaked barley	g/kg	150	150	200
Flaked wheat	g/kg	200	200	150
Dry matter	g/kg	867	868	869
Crude protein	g/kg DM	176	159	151
Ether extract	g/kg DM	20	21	22
NDF	g/kg DM	166	165	165
Ash	g/kg DM	25	22	20
Digestible energy	MJ/kg DM	16.47	16.54	16.58

0.5, 1.0, 2.5, 5, 10 and 20 μg/kg were analysed using extraction and HPLC method The experiment was repeated 5 times. Taking into account dilution and concentration steps, meat spiked samples corresponded to OTA standard concentrations of 0.2, 0.5, 1, 2.5, 5, 10 and 20 μg/l and feed spiked samples corresponded to OTA standard concentrations of 0.8, 2.0, 4.0, 10, 20, 40 and 80 μg/l. The repeatability was tested by analyzing samples of meat and feed spiked with OTA. Meat samples were spiked at the levels of 0.05 μg/kg (corresponding to 0.5 μg/l), 0.25 μg/kg (corresponding to 2.5 μg/l), and 2 μg/kg (corresponding to 20 μg/l). Feed samples were spiked at the levels of 0.5 μg/kg (corresponding to 2.0 μg/l), 2.5 μg/kg (corresponding to 10 μg/l), and 20 μg/kg (corresponding to 80 μg/l). All samples were measured in triplicates on the same day. For the within-laboratory reproducibility test, each of the contamination level was tested in triplicates in seven days. The results of these experiments were used also for the determination of the recovery. Selectivity studies have been expressed as the ability to asses unequivocally OTA in the presence of components which may be expected to be present: it has been evaluated by the comparison of free-OTA vs spiked samples.

Samples preparation

Meat/Backfat/Meat products

A 5 g sample aliquot was homogenized with 5 ml of phosphoric acid 1 M using an Ultra Turrax T25 homogenizer for a few minutes. A 2.5 g aliquot of the homogenate was transferred into a centrifuge tube, extracted with 5 ml of ethylacetate, vortexed for 1 min, shaked for 10 min on horizontal shaker and then centrifuged for 10 min at 3000 rpm. The organic phase was removed, the residue re-extracted, as above, and the organic phases combined. The volume of the organic phase was reduced to approximately 5 ml and back-extracted with 5 ml of $NaHCO_3$ pH 8.4, vortexed for 1 min and centrifuged for 10 min at 3000 rpm. The aqueous extract was acidified to pH 2.5 with $H_3PO_4$85% and briefly sonicated to strip the CO_2 formed. OTA was finally back-extracted into 5 ml ethylacetate, vortexed for 1 min and centrifuged for 10 min at 3000 rpm; the organic phase was evaporated to dryness under nitrogen stream, reconstituted in 250 μl of mobile phase and a 100 μl aliquot injected.

Feed

Ten grams of feed samples were mixed with 40 ml of extraction solution (water/methanol 50/50 v/v). Extraction was done by shaking for 10 min on horizontal shaker and centrifuging at 3000 rpm for 10 min. A 4 ml aliquot of the extract was diluted with 46 ml of phosphate-buffered saline (PBS, pH 7.4). The diluted sample was filtered through Whatman filter paper (Millipore Corporation, Maid Stone, UK). This solution (50 ml) was passed through IAC at flow-rate of 1-2 drops s^{-1}. The column was washed with 20 ml of PBS (1-2 drops s^{-1}). Elution was performed with 3 ml of methanol and acetic acid 98/2 v/v. The elute was evaporated to dryness under nitrogen stream. The residue was redissolved in 250 μl of HPLC mobile phase and injected into HPLC system.

Spiked samples

Samples spiked before extraction were used to check the performance of the extraction and clean-up procedure and to obtain validation parameters. Spiking solutions of OTA were prepared daily by dilution with HPLC mobile phase. For samples of pigs meat, after thoroughly mixing for 30 min, the OTA fortified homogenate was left for at least 2 hours at room temperature to enable equilibration and used to assay the cleaning procedures prior to HPLC analysis. For samples of feed, spiked samples were prepared by adding appropriate volume of the spiking solution of OTA to the feed suspension (described above) and then processed as for meat samples.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (v. 6) software (La Jolla, CA, USA). All data were tested for normality by means of the Kolmogorov-Smirnov test. The data are reported as mean ± standard deviation (SD). The influence of the different rearing system and on OTA concentrations was detected by t-test. The difference in OTA concentrations between fresh and seasoned meat products was detected by t-test. A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSIONS

High-performance liquid chromatography method

Validation parameters of HPLC method are reported in Table 2. The mean OTA recoveries in spiked samples ranged from 79.9 to 89.4% for feeds and from 89.9 to 95.0% for meat. The calculated LOD were 0.0125 and 0.1 μg/kg for meat and feeds, respectively. A calibration curve peak area versus concentration was obtained using the linear squares regression procedure. The OTA linearity in the spiked sam-

Table 2 - Validation parameters for HPLC methods of OTA in meat and feed samples.

Parameters		Meat		Feed
LOD	μg/kg	0.0125		0.1000
LOQ	μg/kg	0.0250		0.2500
r^2		0.9910		0.9920
CV % (intra-day)			CV % (intra-day)	
0.05 μg/kg	n = 3 replicates	5.8	0.50 μg/kg	4.9
0.25 μg/kg	n = 3 replicates	8.9	2.50 μg/kg	9.5
2.00 μg/kg	n = 3 replicates	6.0	10.00 μg/kg	7.8
CV % (inter-day)			CV % (inter-day)	
0.05 μg/kg	n = 21 replicates	9.7	0.50 μg/kg	8.9
0.25 μg/kg	n = 21 replicates	7.6	2.50 μg/kg	8.0
2.00 μg/kg	n = 21 replicates	5.8	10.00 μg/kg	7.0
Recovery %			Recovery %	
0.05 μg/kg	n = 21 replicates	89.9 ± 1.8	0.50 μg/kg	79.9 ± 5.0
0.25 μg/kg	n = 21 replicates	91.2 ± 3.5	2.50 μg/kg	89.4 ± 0.8
2.00 μg/kg	n = 21 replicates	95.0 ± 1.2	10.00 μg/kg	85.9 ± 6.8

Table 3 - OTA concentrations (μg/kg) in analyzed feed samples.

		T1	T2	Significance
Flaked corn	μg/kg	2.03 ± 0.07	2.36 ± 0.42	NS
Flaked protein pea	μg/kg	2.50 ± 0.50	2.60 ± 0.26	NS
Flaked wheat	μg/kg	1.56 ± 0.42	2.12 ± 0.33	NS
Flaked barley	μg/kg	7.17 ± 0.36	7.27 ± 0.68	NS

NS: p > 0.05; T1 beginning the fattening period; T2 end of the fattening period.

Table 4 - OTA concentrations (μg/kg) in analyzed pigs tissues and derived products.

		IND	OTD	Significance
Muscle (fresh)	μg/kg	0.055 ± 0.015[a]	0.078 ± 0.011[a]	*
Muscle (seasoned)	μg/kg	0.088 ± 0.048[b]	0.178 ± 0.031[b]	**
Backfat (fresh)	μg/kg	0.079 ± 0.018[a]	0.085 ± 0.025[a]	NS
Backfat (seasoned)	μg/kg	0.170 ± 0.064[b]	0.204 ± 0.069[b]	NS
Salami	μg/kg	0.058 ± 0.015	0.064 ± 0.004	NS
Mortadella	μg/kg	0.537 ± 0.042	0.558 ± 0.016	NS

NS: p > 0.05; *: p < 0.05; **: p < 0.01. Means followed by different letters differ significantly at p < 0.05.

ples at 5 determinations of 7 concentrations levels was excellent, as shown the correlation coefficient >0.99. The method has shown to be suitable for accurate quantitative determination of OTA in different tissues of pigs and feed samples.

Feed samples

Concentrations of OTA in conventional diet are reported in Table 3. The OTA concentrations found at T1 were not statistically different from those found at T2. None of the feed samples analysed in the present study was contaminated with OTA at a level exceeding the European Commission recommended limit of 50 μg/kg in swine feed[22].

Meat samples

OTA concentrations in tissues and meat products samples are showed in Table 4. OTA was found in all pig's tissues and meat products samples at concentrations lower than the Italian recommended maximum level of 1 μg/kg. Although, OTA content in the diet was much lower than 50 μg/kg, it was enough to determine after lengthy exposure, a final detectable amount in the muscle and backfat. This agrees with previous studies in which a diet naturally contaminated at levels of 0.3 μg/kg for 119 days resulted in final OTA concentrations in muscle of 0.88 μg/kg[23].

OTA concentrations in fresh and seasoned muscle (coppa) of OTD group were significantly higher than those of IND group. OTA concentrations in fresh and seasoned backfat (lard) of OTD group were not significantly different from those of IND group. OTA concentrations in salami and mortadella of OTD group were not significantly different from those of IND group. The highest OTA concentration was found in one mortadella sample of IND group showing a concentration of 0.889 μg/kg very close to the Italian recommended maximum level of 1 μg/kg. This can be related to the type of production of mortadella. Pepper was added only to mortadella samples and not in salami production. The most utilized spices in pork derived products are garlic, anise, cinnamon, cloves, coriander, fennel, nutmeg and pepper. Among these, OTA was reported as a natural contaminant in pepper and nutmeg[24]. Spices, as black pepper, may be able to raise OTA levels in pork derived products. The OTA concentration in mortadella samples could also be the use of organs for its preparation. In fact, several studies in which tissues and organs from pigs exposed to OTA contaminated feed were analysed, have shown that some tissues (such as lung and heart) had higher levels of OTA compared to other matrices such as muscle and fat, that are the main ingredients used in the preparation of products as salami[25-28]. This is the first study reporting OTA detection in mortadella samples, more samples are needed to confirm this hypothesis. Statistically significant differences were observed for OTA content in muscle samples of both OTD and IND groups between fresh and seasoned products (Table 4). Statistically significant differences were observed for OTA content in backfat samples of OTD and IND groups between fresh and seasoned products (Table 4). These results could be related to a concentration effect due to the loss of water during the seasoning of meat products.

CONCLUSIONS

The present study indicate that OTA exposure is a common phenomenon for which limitation is essential to increase controls on foodstuffs taking care of every stage of the production chain, given the growing concern for consumer health. The biggest problem in managing mycotoxin risk is represented by the difficulty of precisely identifying the phases in which contamination can occur, and the great variety of foods on which this can occur. Only a global vision of the problem involving all types of skills, such as agronomic, biological, pathophysiological, chemical and many others could lead to better assessment and management of mycotoxin risk and limit the presence of OTA in food.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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