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Abstract

A total of 172 different salamis were purchased from farms and small salami factories located in four Italian regions (Piedmont, Veneto, Calabria and Sicily), and analysed for the presence of ochratoxin A (OTA). Analysis were performed by high-performance liquid chromatography coupled to a fluorimetric detector (HPLC-FLD). The detection limit (LOD) for the method used was 0.05 µg/kg, while the quantitation limit (LOQ) was 0.20 µg/kg; the average recovery rate was 89.1%. OTA was detected in 22 salamis, and 3 samples exceeded the Italian guidance value for OTA in pork meat (1 µg/kg). In particular, what emerges from this research is the high percentage of spicy salamis among positive samples (68.2%, 15 out of 22), although spicy salamis are only 27.3% of the total number of samples collected and analysed. Red chili pepper contaminated by OTA could be responsible for the presence of the mycotoxin in these spicy salamis. It follow that, also the control of some ingredients used in the manufacture of these meat products, like spices, should not be neglected.

Keywords: mycotoxins; ochratoxin A; salami; spices; contamination; HPLC-FLD.

Introduction

Salami is a typical product of the Italian tradition. Each Italian region produces specific types of salami that can differ on the basis of various criteria: the origin of the meat, the methods of chopping and preparation of the lean and the fatty parts, the ratio between the different parts, the salting and the addition of different spices, the type of casing, the size of the final product, the development of moulds on the casing, the seasoning methods (Armorini et al. 2016). In order to safeguard and enhance these differences often linked with local traditions, producers and/or local authorities have organized themselves in protection consortia to obtain the recognition of their products as PDO (Protected Designation of Origin) or PGI (Protected Geographical Indication) product.

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species of the genera *Penicillium* and *Aspergillus*. Contamination of food commodities, including cereals and cereal products, pulses, coffee, beer, grape juice, dry vine fruits and wine as well as cacao products, nuts and spices, has been reported from all over the world (EFSA 2006). Furthermore, OTA can also contaminate dried meats, blood sausages, meat, milk, infant formula, and baby foods (JECFA 2008). Despite toxin production may occur over a wide temperature range, optimal conditions for ochratoxin production are given by a temperature range between 20-25 °C and a crop moisture content of at least 16% (Völkel et al. 2011).

Ochratoxin A has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic to several species of

animals and to cause kidney and liver tumours in mice and rats (WHO 1996). The International Agency for Research on Cancer (IARC) classified OTA as a possible human Carcinogen (Group 2B) (IARC 1993). Of greatest concern for human health is its implication in Balkan Endemic Nephropathy (BEN), a chronic kidney disease found in South-Eastern Europe (Mantle 2002; Petkova-Bocharova et al. 1988), and in Chronic Interstitial Nephropathy (CIN), a highly similar disease found in North Africa (Abid et al. 2003). A study carried out at European level reports that meat and spices contribute 1% and 8%, respectively, to the mean human dietary intake of OTA (Miraglia and Brera 2002). Another study conducted in the United States indicates that, among the adult population, pork consumption supposes 15.82% of the total OTA intake (Mitchell et al. 2017). OTA may be present in feed and due to the carry-over of the toxin from feed to animal tissues it can be found in some foods of animal origin.

Among farmed animals, pigs are particularly sensitive to OTA, and this mycotoxin plays a special role in the genesis of swine mycotoxic nephropathy (Stoev et al. 2012). In pigs, OTA has been detected in kidney and other edible organs, but in lower concentrations than in whole blood or blood plasma. European legislation has set a guidance value for OTA of 0.05 mg/kg in complementary and complete feedingstuffs for pigs (European Commission 2006), but didn't set maximum OTA levels in meat and meat-based products. Italy is the only European country where a guideline value equal to 1 µg/kg in pork meat and derived products has been set for this mycotoxin (Ministero della Sanità 1999). The presence of OTA in meat products could be the result of either indirect transmission from animals exposed to naturally contaminated feed or, in the case of cured meat products, direct contamination with moulds (Battilani et al. 2007; Bertuzzi et al. 2013). If thermohygrometric conditions are favorable to the settlement of toxigenic moulds, they can grow on the surface during ripening. Consequently, OTA can be produced on the casings and then it can diffuse in the mince (Berni et al. 2017). Furthermore, contaminated spice mixtures used as ingredients in the preparation of salami can be other sources of direct contamination (Pietri et al. 2006). OTA is a relatively stable molecule, and manufacturing procedures such as heating, ripening, drying, and storage have no effects on its reduction in meat products (Amézqueta et al. 2009).

The aim of this study was to obtain data on the occurrence of OTA in artisan salami sampled in four Italian regions, two in the north of the country (Piedmont and Veneto) and two in the south (Calabria and Sicily). For this research, a suitable analytical method based on a sample preparation procedure with immunoaffinity columns (IAC), together with analysis by HPLC with fluorescent detection (HPLC-FLD) was adopted.

Material and methods

Samples

A total of 172 different salamis were purchased, from January 2015 to May 2016, in a random manner from farms and small salami factories located in four Italian regions. In particular, the samples were 52 from Piedmont, 16 from Veneto, 50 from Calabria, and 54 from Sicily. After purchase, the samples were registered and catalogued in the laboratory notebook. From each salami, equal aliquots (cross sections) were sampled in different positions (in the middle and at the two ends) and collected after removing the casing. The aliquots were minced together using a mini grinder (Illico, Moulinex, France) in order to obtain a homogeneous laboratory sample, whose weight was about 40% of the whole salami; finally all the samples were stored at –20 °C until analysis.

Chemicals and reagents

All solvents and reagents were analytical grade or HPLC grade. OTA pure standard used to prepare standard solutions for the validation of the applied methodology was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). OchraTest™ WB immunoaffinity columns from Vicam® (Milford, MA, USA) were used for samples purification. Solvents and reagents 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 obtained from Carlo Erba Reagents (Cornaredo, MI, Italy). The solvents used in the post extraction immunoaffinity clean up (water, methyl alcohol), and all solvents used for HPLC analysis (water, acetonitrile, isopropyl alcohol, acetic acid) were purchased from Mallinckrodt Baker B.V. (Deventer, The Netherlands).

Chromatographic apparatus

For OTA analysis and quantification, a HPLC system consisting of a System Gold Programmable Solvent Module 126 pump (Beckman Coulter, Brea, CA, USA) equipped with an HT 800 L autosampler (HTA, Brescia, Italy) fitted with a 20 µl loop and an 821 FP fluorescence detector (Jasco, Tokyo, Japan) was used. Fluorescence excitation and emission wavelengths were 340 and 460 nm respectively. The system was computer-controlled by a Beckman Coulter 32 Karat Software. The analytical conditions were those developed by Armorini et al. (2016), and optimized for use in this study. Chromatographic separation was achieved in gradient elution mode using two monolithic columns coupled in sequence: a C18 Onyx Monolithic 100 mm x 4.6 mm (Phenomenex, Torrance, CA, USA) and a C18 Chromolith Performance RP-18e 100 mm x 4.6 mm (Merck, Darmstad, Germany). The mobile phase consisted of eluent A containing water/acetonitrile/isopropyl alcohol/acetic acid 1% (79:7:7:7, v/v) and eluent B consisting of acetonitrile. The gradient program started with 58% A and 42% B, reaching 49% B in 3.5 min with linear increase. The flow rate was 1.1 ml/min,

while the sample injection volume was 20 µl.

Sample extract preparation

The extraction of OTA from salami was based on the methods described by Monaci et al. (2004, 2005) and Bozzo et al. (2012) with some modifications. After acidification with 1.5 ml of 1 M phosphoric acid, a 2.5 g aliquot of minced sample was homogenized in 5 ml of ethyl acetate for 3 min using an Ultra-Turrax homogenizer. The sample was centrifuged for 3 min (1900 x g) and the upper organic phase was collected. The extraction procedure was then repeated on the pellet; the organic phase was removed and combined with the one previously collected. The two combined extracts, after being frozen (-20 °C) for 3-4 hours, were centrifuged at 1900 x g for 10 min in order to separate emulsified and suspended components, which were removed. An 8 ml aliquot of the extract (equivalent to 2 g sample) was then measured and reduced to 2 ml with an Univapo rotational vacuum concentrator (Uniequip, Martinsried/Munich, Germany). The organic extract was than back-extracted with 2 ml of 0.5 M sodium bicarbonate solution by mixing on a rotating shaker for 30 min. After centrifugation at 1900 x g for 10 min, the organic phase was removed and the bicarbonate extract was subjected to clean up. The sample clean up procedure was based on the Vicam® protocol for green coffee, modified as follows: an aliquot of 1.5 ml of bicarbonate extract previously diluted with 3.5 ml of PBS buffer was passed through an OchraTest™ WB immunoaffinity column at a flow rate of 1 drop/s. The column was washed with 10 ml of PBS buffer and 10 ml of water, and finally dried for several seconds using draw vacuum. OTA was eluted with 1.5 ml of methanol. The eluate was evaporated by means of Univapo and redissolved in 150 µl of methanol, diluted with 150 µl of water, and after vortexing, injected into HPLC.

Quantification

The analytical method for HPLC-FLD detection of OTA in salami has been validated, including linearity and range, specificity, accuracy and precision, limit of detection (LOD), limit of quantification (LOQ), and recovery. OTA was quantified by external matrix-matched calibration procedure. Calibration solutions for matrix-matched calibration curve were prepared from blank samples spiked directly with OTA standard solutions. Linearity of the method was estimated by analysis of 5 calibration solutions in the range 0.2-2 µg/kg, and it was checked using the coefficient of determination (R^2). Specificity was demonstrated using blank samples and spiked samples to show that the method results were unaffected by the presence of impurities or matrix components. It was checked calculating the mean values (± standard deviation) of the retention time of OTA at the operating conditions of the HPLC system. Accuracy and precision were evaluated via analysis of spiked samples, using 9 determinations over 3 concentration levels in the range 0.2-2 µg/kg (3 concentrations/3 replicates each). Accuracy was expressed as percent error (% er) and precision as the relative standard

deviation (RSD) of the replicate measurements. The limit of detection (LOD) was found by determining the concentration of OTA that produce a chromatographic peak at S/N of 3. The limit of quantification (LOQ) was calculated as the lowest concentration level of the calibration curves of OTA. Recovery was evaluated on fortified blank samples. After extraction, the samples were analysed according to the method described above. The recovery values were calculated by comparing peak areas of the chromatograms obtained and peak areas of pure OTA standard solutions at the same concentration levels. For internal quality control purposes, the unknown samples processed the same day were always accompanied by one or more spiked blank samples. This study was performed according to ISO 9001 requirements (ISO 2008).

Results and discussion

Method validation

Analyses were carried out in four different periods and the validation of the analytical method was performed in the course of each analytical session. The calibration curves showed a linear response over the entire observed range (R^2 always > 0.999). The specificity of the method was satisfactory. In fact, throughout the OTA retention time, no other signals were observed in the non-contaminated samples. Furthermore, retention time for OTA was constant in all the four analytical sessions (5.55 ± 0.06, 5.50 ± 0.05, 5.38 ± 0.04, and 5.16 ±0.07 minutes, respectively). The run time was 7 minutes. Figure 1 shows representative chromatograms of a blank sample (A), and a naturally contaminated sample at 5.66 µg/kg level (B). For each analytical session, 3 calibration standards at concentration of 0.4, 1, and 1.5 µg/kg were prepared and analysed in three different days. RSD and % er never exceeded 6.1% and 4.0%, respectively. LOD and LOQ estimated for all analysed meat products were 0.05 and 0.20 µg/kg, respectively, far below the Italian guideline value of 1 µg/kg. Recovery experiments were performed at 3 spike levels, and for each concentration, three measurements were done. The results are shown in Table 1, and the overall average recovery was 89.1%. Analytical performance results proved that the method adopted was fit for the quantitative determination of OTA in these meat products, and generated reliable results.

Occurrence of ochratoxin A in salami samples

As reported above, in this study 172 samples of salamis were analysed, 22 of which were positive for the presence of OTA, representing 12.8% of the total number of samples. Salami samples and OTA concentrations are reported in Table 2. In particular, 3 of these positives (representing 1.7% of the total number of samples and 8.6% of the positives only) exceeded the guidance value of 1 µg/kg established for OTA by the Italian Ministry of Health. The maximum

concentration detected was 5.66 µg/kg, while the average concentration of the positive samples was rather low (0.51 µg/kg). The distribution of the concentration levels detected (Figure 2) shows that more than 50% of the positive samples was in the concentration range 0.10-0.19 µg/kg. Thus, the overall results of the survey suggest that these typical regional salamis made with the traditional, non-industrial production method, as regards contamination by OTA, can be considered safe. Additional significant data emerge analysing the results by region of production (Table 3) and type of salami, as reported below.

Calabrian salamis show the highest percentage of positive samples (26%). In particular, 12 out of 13 of these positives are spicy salamis (6 "Nduja" and 6 "Piccante"), and all these salamis contain red chili pepper. This ingredient can be present in varying amounts, from about 8% by weight in "Piccante", to 20-30% by weight in "Nduja". If we only consider the spicy salamis, the percentage of positives is 36.4% (12 out of 33), that is much higher than in the group of sweet salamis (5.9%, 1 out of 17). Furthermore, 6 out 8 samples of "Nduja" (75%) were positive for OTA. These data leads to the assumption that the contamination by OTA of these samples could be due to pepper, that is known among different spices to be one of the most highly and frequently contaminated by this toxin (Miraglia and Brera 2002). Contamination by OTA of spices and pepper in particular, has been reported from all over the world (EFSA 2006). Some studies carried out in South America and India even suggested that a high consumption of red chili pepper would be a risk factor in the development of gallbladder cancer because of the high incidence of contamination by OTA of pepper (Ikoma et al. 2015, 2016).

With regard to "Nduja", one of the causes of contamination could also be the use of organs such as lung and heart for the preparation of the dough. In fact, several experiments in which tissues and organs from pigs exposed to OTA-contaminated feed were analysed, have shown that 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 salamis (Altafini et al. 2017; Perši et al. 2014; Pleadin et al. 2014, 2016).

Piedmontese salamis show a low percentage of positive samples (7.7%) and all these positives come from the same production plant. We hypothesized that the contamination of these salamis may be due to factors closely linked to the production plant rather than the type of product. In this case, the ripening rooms are tufa soil caves where there is not a temperature and humidity regulation system. In these environments, it would be possible the development of OTA producing moulds. This hypothesis would be supported by the fact that the other samples coming from the same farm, negative for the presence of OTA, were fresh salamis or salamis that have been matured for a short period (less than seven days).

Sicilian salamis show a low percentage of positive samples (9.3%), but it is to be noted that, of 5 positives, 4 were from the same production plant. It is therefore clear the problem of ochratoxin A contamination in this site of production, especially because the positives were 4 on a total of 10 salamis that were sampled in the site. Furthermore, 3 out 4 of these positives were spicy salamis, and it is possible that the contamination may be due to the presence (about 6% by weight) of contaminated red chili pepper**.** However, of these 4 positives, the higher concentration of OTA (1.03 µg/kg) was detected in a sweet salami, and therefore the causes of contamination may be different.

Venetian salamis were negative for the presence of OTA. These salamis were sampled in the same farm, indicating that in the site of production, at least in the sampling period, there were not problems of direct or indirect contamination by OTA.

Finally, focusing on the question of salamis containing red chili pepper, considering all the spicy salamis from all regions (2 from Piedmont, 33 from Calabria, and 12 from Sicily), the total number of positive for the presence of OTA is 15 out of 47 (31.9%), while the percentage of spicy salamis over the total number of positives is 68.2% (15 out of 22), a very high value, especially given that spicy salamis are only 27.3% of the total number of samples analysed. However, as all the salamis included in this study were sampled only in their final form, ready for consumption, the exact cause of their contamination (contaminated raw materials, drying, ripening and storage conditions, or else) remains undetermined. It is important to report that the external appearance of all the salamis examined, and thus also the positives, was normal without damages of casing. In fact, such damages may result in the entry of OTA produced by surface moulds into the product causing its significant contamination (Pleadin et al. 2015). Also the appearance on cutting was normal, no existence of cracks was underscored, no signs of spoilage were found in any sample, and the organoleptic characteristics (particular smell and flavour) were very good.

Several studies have been carried out to monitor the presence of OTA in pork meat-derived products. Monaci et al. (2005) described a procedure to detect OTA, which was applied to the screening of 30 pork raw sausages collected from local retailers in Southern Italy. Forty-three percent of the analysed samples were found contaminated with OTA at levels between 0.06 and 0.4 µg/kg. A survey of 160 sausages produced in Northern Italy was performed by Iacumin et al. (2009). In these samples, OTA concentration was determined on the casings and inside the meat at a depth of 0.5 cm. OTA was detected on the casing in 72 samples (45% of positives) in the range 3-18 µg/kg, but not inside the meat. This result means that the mycotoxin did not diffuse through the casing into the meat and raw meat did not contain OTA. In a survey carried out by Dall'Asta et al. (2010), 110 different types of dry-cured ham from the Italian market were examined. OTA was detected on the surface portion and in the inner core in 84 and 32 samples, respectively. The maximum detected levels were 12.51 µg/kg (outer portion) and 4.66 µg/kg (inner portion).

A study to determine the possible presence of OTA, aflatoxin B1 (AFB_1) and citrinin (CIT) in various Croatian fermented meat products (n=90), was carried out by Markov et al. (2013). OTA was detected in 58 samples (64.44% of positives) in the range 1.23-7.83 µg/kg and this mycotoxin has proved to be the predominant contaminant, while only 10% of the samples were contaminated with AFB_1 and 5.55% with CIT.

Pleadin et al. (2014) investigated the occurrence of aflatoxin B_1 and OTA in 410 traditional meat products produced by households situated in different Croatian regions. Considering only OTA contamination in the dry-fermented sausages (n=208), the results showed a low percentage of positive samples (6.7%) in the range 0.95-5.10 µg/kg. Armorini et al. (2016) reports about 50 samples of artisan salamis purchased in Veneto (Italy) and analysed for the presence of OTA. The mycotoxin was detected in 5 samples, and the very high concentration observed in one sample (103.69 µg/kg) proved that a high contamination by OTA is also possible in this type of product. The findings of this preliminary investigation suggested to extend the research in other regions of Italy where an ancient tradition in the production of typical salamis is present, and it has resulted in the present paper.

In another monitoring study conducted from 2013 to 2015 in Northern Italy, OTA was detected at a concentration level > 1 µg/kg in 13 out 133 samples of traditional salamis (9.8% of the total count) (Merla et al. 2018).

The results of the abovementioned studies generally show non-negligible percentages of positives and concentration levels of OTA often above the Italian guideline value set for pork meat and derived products. Therefore, in pig meat products, the risk for this type of intoxication cannot be underestimated, although it is lower than in cereals and others products of plant origin.

As regard the causes of OTA contamination of dry-cured meats and dry-fermented products, several studies have shown that this type of contamination would be largely dependent on the characteristics and the environmental conditions of the manufacturing plants, particularly with reference to temperature, relative humidity, and environmental mycoflora composition (Alapont et al. 2014; Battilani et al. 2007; Iacumin et al. 2009; Rodríguez et al. 2015). Some authors reported that indirect transmission of OTA from animals exposed to contaminated feed to pork products occurs rarely (Iacumin et al. 2009; Pietri et al. 2006). Hence the importance of ensuring first of all the control of the environmental conditions of the manufacturing plants, without overlooking the entire chain of meat production “from feed to food”.

In conclusion, the present study on the occurrence of OTA in salamis shows a relatively small percentage of positive samples and only 3 samples above the Italian guidance value for OTA in pork meat. The survey further reveals a rather high percentage of positive samples among spicy salamis. It is reasonable to assume that red chili pepper contaminated by OTA could be responsible for the presence of the mycotoxin in these samples. It follow that, also the control of some ingredients used in the manufacture of these meat products, like spices, should not be neglected.

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Conflicts of interest None.

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Fig. 1 Chromatograms obtained on the IAC purified extracts of (A) salami blank sample, and (B) a salami found naturally contaminated with OTA at 5.66 µg/kg level

Fig. 2 Distribution of OTA levels detected in the positive samples

Table 1 Recovery of the HPLC-FLD method for analysis of OTA in the four groups of salamis analysed

Analytical session/group of salamis	Conc. level (µg/kg)	Recovery (%)[a]	RSD (%)[a]
1st session/Sicilian salamis	0.4	95.68%	4.06%
	1	96.95%	1.90%
	1.5	90.78%	0.96%
2nd session/Calabrian salamis	0.4	87.90%	4.12%
	1	86.63%	7.32%
	1.5	85.56%	2.76%
3rd session/Piedmontese salamis	0.4	90.05%	5.76%
	1	86.62%	2.84%
	1.5	87.87%	5.61%
4th session/Venetian salamis	0.4	87.27%	5.27%
	1	86.60%	0.23%
	1.5	87.20%	2.47%

[a] number of replicates: 3

Table 2 Samples of salami tested positive for OTA

Ref.	Region	Type of salami	OTA (µg/kg)	Notes
48 P [a]	Piedmont	Cacciatorino	0.13	Hunter salami
50 P [a]	Piedmont	Salame Crudo	0.15	Raw salami
51 P [a]	Piedmont	Salame Crudo	5.66	Raw salami
52 P [a]	Piedmont	Salame Crudo	1.51	Raw salami
3 C [b]	Calabria	Nduja	0.08	Typical spicy Calabrian salami
5 C [b]	Calabria	Nduja	0.14	Typical spicy Calabrian salami
18 C [c]	Calabria	Nduja	0.13	Typical spicy Calabrian salami
19 C [c]	Calabria	Nduja	0.12	Typical spicy Calabrian salami
20 C [c]	Calabria	Nduja	0.24	Typical spicy Calabrian salami
35 C	Calabria	Salsiccia Piccante	0.16	Spicy salami
36 C	Calabria	Salsiccia Piccante	0.16	Spicy salami
37 C [d]	Calabria	Salsiccia Piccante	0.10	Spicy salami
39 C	Calabria	Salsiccia Piccante	0.12	Spicy salami
40 C [d]	Calabria	Salsiccia Piccante	0.20	Spicy salami
41 C	Calabria	Salsiccia Piccante	0.14	Spicy salami
42 C [d]	Calabria	Salsiccia Dolce	0.07	Sweet salami
49 C	Calabria	Nduja	0.62	Spicy salami
16 S	Sicily	Salame Nostrano	0.07	Traditional salami
44 S [e]	Sicily	Salame Piccante	0.07	Spicy salami
46 S [e]	Sicily	Salame Piccante	0.09	Spicy salami
49 S [e]	Sicily	Salame Piccante	0.19	Spicy salami
50 S [e]	Sicily	Salame Torciglione	1.03	Typical salami

[a] samples 48 P, 50 P, 51 P, and 52 P from the same production plant
[b] samples 3 C and 5 C from the same production plant
[c] samples 18 C, 19 C, and 20 C from the same production plant
[d] samples 37 C, 40 C, and 42 C from the same production plant
[e] samples 44 S, 46 S, 49 S, and 50 S from the same production plant

Table 3 Occurrence of OTA in salamis

Samples	N° positive/ N° total samples	% positive	Mean (µg/kg)[a]	Median (µg/kg)[a]	Standard Deviation (µg/kg)[a]	Range (µg/kg)
Salamis (all types)	22/172	12.8	0.51	0.14	0.32	0.07-5.66
Piedmontese salamis	4/52	7.7	1.86	0.83	2.61	0.13-5.66
Venetian salamis	0/16	0.0	/	/	/	/
Calabrian salamis	13/50	26.0	0.18	0.14	0.14	0.07-0.62
Sicilian salamis	5/54	9.3	0.29	0.09	0.42	0.07-1.3

[a] values calculated considering only the positive samples

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