

Levels of aflatoxin B1 and ochratoxin A in Croatian and Slovenian traditional meat products

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Scientific paper

Abstract

Mycotoxins aflatoxin B1 (AFB₁) and ochratoxin A (OTA) present a common source of contamination of meat products. In this study, the three types of Croatian and Slovenian traditional meat products (n = 18) from the categories of dry-cured products, fermented sausages and dry-cured bacon, were analyzed on the levels of mycotoxins AFB₁ and OTA. After determination of basic chemical composition of the products, the levels of mycotoxins were investigated by use of validated quantitative ELISA method. The highest level of OTA, with a similar concentrations, was determined in "Dalmatinski pršut" (2.75 µg/kg) and "Kraški pršut" (2.86 µg/kg). In the same products, AFB₁ was determined with values of 1.16 µg/kg and 1.39 µg/kg, which don't represent the levels significantly higher (p < 0.05) than the applied analytical method limit of detection. In the other products AFB₁ was not detected. The results indicate a lower level of contamination of traditional meat products with AFB₁ in comparison to OTA, which was not detected only in the Slovenian product "Zgornjesavinjski želodec". Determined presence of AFB₁ and OTA in traditional meat products can be derived from contaminated raw materials as muscle and fat, and spices used in their production, but also as consequence of direct product contamination due to the production of these mycotoxins from moulds that spontaneously overgrown the surface of fermented meat products during their ripening.

Keywords: contamination, aflatoxin B1, ochratoxin A, Croatian and Slovenian traditional meat products

Introduction

Mycotoxins as toxic secondary metabolites of moulds pose a threat to food security worldwide. Due to possible contamination of natural grains that are used in the production of food and feed, there is a possibility of mycotoxins entrance in human food chain (Walker and Larsen, 2005; Khoury and Atoui, 2010). Literature data show that products of animal origin, such as meat and meat products, may also contribute to the entry of mycotoxins in food, whether as a result of indirect transmission from domestic animals, which are used for the production of food of animal origin, exposed to contaminated feed materials and compounds (carryover effect), but also through the mixture of spices used in their production (Pleadin et al., 2013; Perši et al., 2014) or direct contamination with moulds which under certain conditions can produce mycotoxins (Gareis and Wolff, 2000; Duarte et al., 2010).

The aflatoxin B1 (AFB₁) and ochratoxin A (OTA) are common contaminants of meat products, and literature data show that technological operations in the production of these types of foods, such as thermal processing, curing, drying, ripening and storage, have not a significant impact on reducing the level of these expressive toxins in the finished meat product (Bullerman and Bianchini, 2007; Amezcua et al., 2009; Kovačević et al., 2014a; Pleadin et al., 2014a). The AFB₁ represents the most potent carcinogen in mammalian liver and is classified by the International Agency for Research on Cancer (IARC) in group 1 as evidenced human carcinogen (IARC, 2002). The presence of AFB₁ in animal nutrition may cause reduced production of food of animal origin, causing a num-

ber of toxic effects in different animal species (Richard, 2007). Given that researches have indicated a widespread distribution of OTA, and with respect to its nephrotoxic properties and other toxic effects in humans and animals (Creppy, 1999; JECFA, 2001), this toxin is included in group 2B as possible human carcinogen (IARC, 1993). Among domestic animals, pigs are particularly susceptible to the toxic effects of mycotoxins (Gareis and Wolf, 2000; Lusky et al., 1993; Pietro et al., 2006), while less susceptible are ruminants, since in the rumen implement their enzymatic degradation in the less toxic metabolites (EFSA, 2004; Duarte et al., 2010).

OTA was determined in elevated levels in meat products produced from contaminated raw materials (Gareis and Scheuer, 2000; Pleadin et al., 2013; Perši et al., 2014), and significant level was found in the dry-cured meat products from the market (Pfohl-Leszkowicz and Manderville, 2007; Dall'Asta et al., 2010). It is known that AFB₁ may be consequently present in meat and meat products if the animals were fed with diet that had significant levels of AFB₁ (Richard, 2007; Herzallah, 2009). Several studies have shown that moulds of *Penicillium* and *Aspergillus* genera, isolated from the surface of meat products from the category of fermented sausages and hams, under certain conditions of production, such as temperature, water activity, damage of cover, presence or absence of skin (ham) or cracks, and storage of the products, produce these mycotoxins (Iacumin et al., 2009; Asefa et al., 2011; Rodríguez et al., 2012).

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Markov et al. (2013) found the OTA contamination of meat products from the Croatian market and very low levels of AFB₁ in fermented meat products. However, later in Croatia was determined extremely high AFB₁-contamination of maize and feed compounds produced from such contaminated maize used in breeding of animals (Pleadin et al., 2014b; Pleadin et al., 2015), which certainly could contribute significantly to contamination of meat products. The results of numerous previous studies have pointed to the possible high levels of contamination of food and feed with AFB₁ and OTA in many countries of the world, as a result of inadequate control of production and storage conditions, indicating the necessity of prevention and continuous control of these extremely toxic mycotoxins.

In this investigation, contamination with mycotoxins AFB₁ and OTA were studied in different types of Croatian and Slovenian meat products from the category of dry-cured products, fermented sausages and dry-cured bacon, produced by traditional technologies which include a long-term process of ripening with temperature and relative humidity conducive to moulds growth on the surface of the product.

Materials and methods

Samples and sample preparation

The study was conducted on six types of traditional meat products (three originating from Croatia and three from Slovenia) from the category of dry-cured products („Dalmatinski pršut“, „Kraški pršut“, „Kraški zašink“), fermented sausages (Istrian sausage, „Zgornjesavinjski želodec“) and dry-cured bacon (Slavonian bacon). From each type of samples from the same manufacturer, three parallel samples (in total 18 samples) were taken and analyzed on the basic chemical composition and mycotoxins AFB₁ and OTA.

All mentioned samples/products were produced from the first and second categories of meat (excluding offal), according to traditional recipes and technologies for „Dalmatinski pršut“ and „Kraški pršut“, as well as products protected with geographical indication at the national level or the „Kraški zašink“ and „Zgornjesavinjski želodec“ as the products protected with geographical indication at EU level described in details in the Specifications (2012a,b; 2013a,b), and for Istrian sausage and Slavonian bacon described in literature (Kovačević, 2001; Kovačević, 2014b; Bratulić et al., 2011). The raw products were subjected to the processes of fermentation, drying and prolonged ripening in darkened chambers („Dalmatinski pršut“ and Slavonian bacon were only smoked). Ripening takes place on average temperatures 12 - 18 °C and a relative humidity of 70-80%, with a slow air flow, for a period longer than 12 months („Dalmatinski pršut“ and „Kraški pršut“), more than three months („Kraški zašink“, „Zgornjesavinjski želodec“, Istrian sausage) and 1-2 months (Slavonian bacon).

Representative samples of meat products for analysis were prepared in accordance with ISO 3100-1:1991. The samples were homogenized at 5000-6000 rpm for 20 s using a homogenizer Grindomix GM 200 (Retch, Ger-

many) and stored in plastic vials at 4 °C until determination of the chemical composition parameters and levels of mycotoxins.

Chemicals and reagents

AFB₁ standard solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and OTA (Acros Organics, Geel, Belgium) used in the validation process were prepared as a stock and working solutions with concentrations of 10,000 µg/L and 10 µg/L, respectively for both analytes, and stored at 4 °C until ready to perform the validation process.

ELISA kits used for the determination of AFB₁ (Aflatoxin B₁; Cat. No. R1211) and OTA (Ochratoxin A; cat. No. R1311) were purchased from R-Biopharm (Darmstadt, Germany). Each kit contains a microtitre plate with 96 wells coated with AFB₁/OTA antibodies, AFB₁ methanol/water standard solution (0, 1, 5, 10, 20, and 50 µg/L) or OTA aqueous standard solutions (0, 50, 100, 300, 900, and 1,800 ng/L), peroxidase conjugate AFB₁/OTA, substrate/chromogen solutions, stop solutions and dilution/washing buffers.

All other chemicals used in the analysis were of analytical grade (Kemika, Zagreb, Croatia). Ultra-pure water was obtained by means of Direct-Q 3 UV (Merck, Darmstadt, Germany).

Validation of analytical methods

For the purpose of validation of analytical methods for determination of the chemical composition the repeatability, within-laboratory reproducibility and trueness were determined, according to the guidelines of the Official Gazette on the performance of analytical methods and the interpretation of results (OG 2/2005). The reference material of canned meat T0149 (FAPAS, England) with indicated values of water content, total fat and protein, was used.

ELISA methods for the determination of AFB₁ and OTA, validated through the determination of limits of detection (LOD) and limit of quantification (LOQ), obtained by adding three or ten values of standard deviation to mean value of ten analyzed fermented domestic sausage samples (AFB₁, OTA < LOD). Method recovery was determined by spiking of the samples at three different levels, each level with six replicates. To determine reproducibility, the same steps were repeated as for the determination of the recovery under the same conditions of the analytical method and the shortest possible period of time to repeat the process two more times.

Determination of basic chemical composition

Water content was determined by gravimetric analysis (ISO 1442:1997) by use of thermostat Epsa 2000 (Ba-Ri, Croatia) at 103 °C. Content of the total proteins was determined by the method of Kjeldahl (HRN ISO 937:1999) with the use of the block for the destruction Unit 8 Basic (Foss, Sweden) and the automated device for the distillation and titration Kjelttec 8400 (Foss, Sweden). Total fat was determined by Soxhlet (HRN ISO 1443:1999) by digestion of the samples by acid hydrolysis, followed

by extraction of the fats by means of petroleum ether on Soxtherm 2000 Automatic (Gerhardt, Germany) and drying in an oven Epsa 2000 (Ba-Ri, Croatia). Ash content was determined according to ISO 936:1998, burning the samples at 550 °C in the furnace LV9/11/P320 (Nobert, Germany).

Results of analysis are expressed as the mean of three parallel determinations per sample, in percent of weight (%), with an accuracy of 0.01%

Methods of AFB₁ and OTA extraction

AFB₁: Three grams of meat product sample and 20 mL of acetonitrile (50%) were shaken on a head-over-head shaker for 90 min. After centrifugation (15 min, 5000 rpm, 10 °C), 3 mL of the supernatant was diluted with 12 mL of deionized water and purified using an Isolute[®] Myco Columns (60 mg/3 mL, Biotage, Sweden). Columns were initially balanced by use of 2 mL of acetonitrile and water. Then, on the columns 3 mL of the sample was applied and washed with 6 mL of water and 10% acetonitrile. Columns were then dried for 10 min under the maximum vacuum and washed with 2 mL of 0.1% formic acid in acetonitrile and 2 mL of methanol. The obtained eluate was evaporated under a stream of nitrogen and dissolved in 0.5 mL of methanol/water (35/65), and the solution was applied to the wells of ELISA kit.

OTA: In 1 g of sample, 6 mL of ethyl acetate and 0.5 mL of 1 M H₃PO₄ were added, shaken on minishaker, and centrifuged at 3000 rpm at room temperature. Then, the ethyl acetate layer was transferred by decantation and extraction procedure was repeated with the further addition of 6 mL of ethyl acetate. After centrifugation, the supernatant was coupled to the first part of ethyl acetate and 3 mL of 0.26 M NaHCO₃ was added. The layers were thoroughly mixed and centrifuged, and 0.8 mL of the lower aqueous phase was transferred to a test tube and heated in a water bath at 100 °C for 5 min. The samples were gently shaken, cooled to room temperature and diluted with 0.2 mL of 0.225 M HCl and 1 mL of 0.13 M NaHCO₃. The resulting solution was used to the wells of ELISA kit.

Determination of AFB₁ and OTA

ELISA tests for both analyzed mycotoxins were performed completely according to kits manufacturer's instructions, with the use of automatized analyzer Chemwell 2910 (Awareness Technologies, Inc., USA). Standards and samples were analyzed in duplicates. After addition of all components of the kit, reaction was stopped by addition of 100 µL of stop solution, and the absorbances of the wells were measured at 450 nm. When calculating the level of AFB₁ and OTA in meat products, the results obta-

ined from the calibration curve were multiplied by the appropriate dilution factor and increased for the value of analytical method recovery determined through validation process.

Statistical data analysis

Statistical analysis was performed using Statistica Ver. 10 software (StatSoft Inc., Tulsa, OK, 1984-2011, USA). Statistically significant differences were analyzed on the level of 95% (p = 0.05).

Results and discussion

Studies suggest very frequent mycotoxin contamination of pork and consequently finished meat products, stating that in order to prevent and ensure products safety, continuous monitoring of these contaminants in meat products is required (Chiavari et al., 2002; Pietri et al., 2006; Pleadin et al., 2013). While mycotoxins in the meat occur primarily as a result of indirect transmission via naturally contaminated feed, contamination of final meat products is often the result of the used recipe, in particular the origin of meat and the use of edible tissue, blood and spices in their manufacture, which may be contaminated with mycotoxins (Gareis and Scheuer, 2000). Also, studies show that for the formation of mycotoxins and other conditions of manufacture and products storage are important (Dall'Asta et al., 2010; Asefa et al., 2011; Rodríguez et al., 2012).

In this part of Europe traditional meat products are mainly of pork, whether of industrial origin or from rural households and family farms. These products are widely consumed by the consumers and offered on the market, while about contamination of these products with mycotoxins there is no enough available data. At the same time, in the last decades the data shown the OTA contamination of grains and feed in these areas (Pepeljnjak et al., 2008), and recent data indicate a significant contamination of maize and feed with AFB₁ (Pleadin et al., 2014b; Pleadin et al., 2014c; Pleadin et al., 2015). The published results of these studies suggest that obtained levels of feed materials and their contamination may consequently result in significant contamination of meat and meat products if they are produced from animals in which diet such contaminated materials were used.

In this study, mycotoxins AFB₁ and OTA in selected traditional Croatian and Slovenian pork meat products, were investigated. Firstly, basic chemical composition of these products were determined. Used analytical methods were previously validated, and results of validation procedures are shown in Tables 1 and 2.

Table 1. Results of validation of analytical methods for determination of basic chemical composition

Validation parameter	Mean ± SD (%)		
	Water (n=6)	Total fat (n=6)	Total proteins (n=6)
Assigned value	69,5±0,98	2,50±0,37	18,22±0,66
Repeatability	69,2±0,25	2,51±0,11	18,37±0,16
Reproducibility	69,4±0,10	2,52±0,08	18,42±0,14
Trueness	69,4±0,08	2,52±0,13	18,35±0,10

The values determined by the validation of methods were compared with the criteria of the Official Gazette (OG 2/2005) and repeatability criteria defined in used ISO standards. By comparing the obtained results with the assigned values or criteria from standards, methods can be considered as acceptable for the determination of basic chemical parameters of meat products.

Investigations showed that in farm animals after dietary exposure to mycotoxins, there were differences in the accumulation of AFB₁ and OTA in tissues, also in muscle and adipose tissue (Gareis and Scheuer, 2000; Pleadin et al., 2013). Therefore, in this study initially was determined the chemical composition of selected Croatian and Slovenian traditional meat products (Table 3), and

Tablica 2. Validation of ELISA methods for determination of AFB₁ and OTA in meat products^a

Analit	LOD	LOQ	Spiking level (µg/kg)	Recovery (%)	CV (%)	Repeatability (%)	CV (%)
AFB ₁	1,04	1,90	2,0	83,4	6,5	81,6	9,2
			5,0	91,3	8,1	90,4	11,2
OTA	0,95	1,77	2,0	89,5	7,9	88,2	10,5
			5,0	93,1	8,8	92,4	12,8

^a validation was performed on control samples of fermented domestic sausages (AFB₁, OTA < LOD)

Results of ELISA method validation used for determination of mycotoxins in various meat products resulted in a mean value of recovery of 87.4% for AFB₁ and 91.3% for OTA and repeatability of 86.0% for AFB₁ and 90.3% for OTA. Mean values of the coefficient of variation (CV) obta-

then the levels of mycotoxins AFB₁ and OTA (Table 4).

The values of the chemical composition are typical for these types of Croatian and Slovenian meat products and comparable with the published literature (Kovačević, 2001; Kovačević, 2014b; Prevolnik et al., 2012; Andronikov

Table 3. Mean values of basic chemical composition of analysed Croatian and Slovenian traditional meat products

Meat product	Maseni udio (%)			
	Water (n=3)	Total fat (n=3)	Total proteins (n=3)	Ash (n=3)
"Dalmatinski pršut"	44,49	11,38	35,72	8,35
Istrian sausage	23,85	39,28	31,95	4,96
Slavonian bacon	15,12	55,63	23,14	6,07
"Kraški pršut"	45,13	15,80	32,63	6,48
"Kraški zašink"	47,81	18,41	27,93	5,90
"Zgornjesavinjski želodec"	35,39	26,20	32,46	6,01

ined in determination of recovery were less than 10% and for repeatability less than 15%. Validation results are consistent with the results of earlier studies, which have also shown that the ELISA method can be used as an effective analytical method for the determination of mycotoxins in meat and meat products (Matrella et al., 2006), with a high correlation with results of HPLC-FLD method as a confirmatory method, also used in our earlier studies (Pleadin et al., 2013; Perši et al., 2014).

et al., 2013) and Specifications (2012a,b; 2013a,b) of these products for registration of a geographical protection. The share of total fat ranged from a minimum quantity in "Dalmatinski pršut" (11.38%) and "Kraški pršut" (15.80%) to the highest in Slavonian bacon (55.63%) and Istrian sausage (39.28%). A high proportion of the total protein (> 20%) in all traditional products shows that this present is a high-quality meat products with significant differences ($p < 0.05$) in their composition i.e. used recipes.

Table 4. Levels of AFB₁ and OTA determined in Croatian and Slovenian traditional meat products

Meat product ^a	Levels of mycotoxins (µg/kg)					
	AFB ₁			OTA		
	Mean ^b	SD	Max	Mean ^b	SD	Max
"Dalmatinski pršut"	1,09	0,10	1,16	2,04	0,45	2,75
Istrian sausage	n.d.	n.d.	n.d.	1,48	0,37	1,81
Slavonian bacon	n.d.	n.d.	n.d.	1,07	0,08	1,12
"Kraški pršut"	1,12	0,12	1,39	2,30	0,34	2,86
"Kraški zašink"	n.d.	n.d.	n.d.	1,54	0,12	1,79
"Zgornjesavinjski želodec"	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

^a per product type three samples were analyzed; ^b the mean value of positive samples (> LOD); n.d. – not detected

The highest OTA level was determined in "Dalmatinski pršut" (2.75 µg/kg) and was similar to level obtained in "Kraški pršut" (2.86 µg/kg). Certain values are two to three times higher than the maximum recommended level defined in some EU countries, such as in Italy, where the maximum recommended value, as prescribed by the Italian Ministry of Health, for OTA in meat and meat products is 1 µg/kg (Duarte et al., 2010). The values of the maximum permitted or recommended levels are not defined by the regulations not in Croatia nor Slovenia. OTA was detected in all products, except for Slovenian products "Zgornjesavinjski želodec".

However, with regard to the products in which OTA was determined, but not in a significant levels of contamination, as that present OTA values greater than 10 µg/kg described in some studies (Iacumin et al., 2009), the level of contamination determined in our study can be attributed to contaminated raw materials used in manufacture, i.e. carryover effect in edible tissues of pigs. By comparison with the established chemical composition, these types of products from the category of hams, have the lowest level of total fat, and the largest proportion of muscle tissue, which is also consistent with the literature data that shown the significant accumulation of OTA in muscle compared to adipose tissue (Gareis and Scheuer, 2000; Pleadin et al., 2013).

In 56% of various meat products sampled from the Italian market, Zannotti et al. (2001) determined the level of OTA above 1 µg/kg. Chiavari et al. (2002) reported the presence of OTA in 42 samples of ham, both in the middle and at the end of the ripening period, with OTA levels higher than recommended in 15 samples and with the highest level of 2.3 µg/kg. The occurrence of OTA in meat and meat products with range from 0.1 to 3.4 µg/kg, that also present the values related to our research data, was associated with the use of contaminated raw materials as pig blood, kidney and liver or contaminated spices (Gareis and Scheuer, 2000). The level of OTA in prosciutto was greater than 1 µg/kg in 5 samples (17%), and 10 µg/kg in the two samples (7%), and the authors concluded that the presence of OTA in these products should be monitored (Pietri et al., 2006). In Croatia, the largest concentration of OTA was determined in winter salami (7.83 µg/kg), while in prosciutto of unknown origin the largest value was 1.03 µg/kg (Markov et al., 2013).

The occurrence of moulds that under certain optimum conditions can produce AFB₁ and OTA is generally characteristic of the dry-cured meat products during the production phase of drying and ripening (Asefa et al., 2010). Moulds that overgrown the surface of prosciutto include several species, mainly from the *Aspergillus* and *Penicillium* genera (Dall'Asta et al., 2010). Some authors state that only direct contamination with moulds during ripening of prosciutto can explain high levels of OTA in certain samples. The emergence of OTA on the surface of the meat products in the performed studies was a result of the production of *Penicillium nordicum* (Battilani et al., 2007; Sorensen et al., 2008) and *Penicillium verrucosum* during the ripening of prosciutto (Spotti et al., 2001; Iacumin et al., 2009).

Moulds of the *Penicillium* genus are used as a typical

starter cultures for the production of fermented sausages in Europe, also in Croatia and Slovenia. These cultures are surface-inoculated in the production of dry sausages because of their impact on improving the flavor, texture and appearance. However, Frece et al. (2010) in Croatia isolated *Aspergillus* sp. and *Penicillium* sp. from the surface of domestic sausages and link them with the production of OTA. In this study, determined OTA levels in products with high quantity of fat tissue, such as Istrian sausages, can be attributed to this type of contamination.

In the products with the highest level of OTA, and AFB₁ was also determined, in the level of 1.16 µg/kg in "Dalmatinski pršut" and 1.39 µg/kg in "Kraški pršut". However, certain levels don't represent a significantly higher ($p < 0.05$) values than the limit of detection of the applied analytical method. AFB₁ in other products was not detected, indicating to lower level of contamination of traditional meat products with AFB₁ as compared to the OTA. Published literature data show that the risk of contamination of dry-cured meats and fermented sausages with AFB₁ is minimal, mainly because of low rates of carryover effect of mycotoxins on edible tissues, given that the primary target organ of AFB₁ is liver. In muscle tissue, only low levels of AFB₁ was found, often below the limit of detection of used analytical method (Beaver et al., 1990; Bintvihok et al., 2002), indicating a very intense AFB₁ metabolism in the liver.

Previously published data have pointed out the possible "diffusion" of mycotoxins from the surface to the interior of meat products, as a result of cover damage, and an increased risk of mycotoxin contamination for the products that because of improper stuffing contain cracks or have excessive moldy cover (Kovačević, 2014b; Kovačević et al., 2014c). The authors describe the importance of controlled production conditions, such as optimal temperature, pH, water activity, the addition of salts and other relevant parameters of production and storage of meat products which, if not controlled, may favor the production of moulds and consequent formation of mycotoxins (Asefa et al., 2010; Asefa et al. 2011). Therefore, in order to prevent possible consequences for human health, food and feed production necessary should be based on the principles of good agricultural and manufacturing practices and analysis of critical control points (HACCP) during the technological process of manufacture and storage. Likewise, it is necessary to ensure the systematic control of the presence of these toxins in food of animal origin and to define the national maximum recommended levels for different categories of food, including meat products.

Conclusion

Occasional contamination of traditional meat products from the category of dry-cured meat products, fermented sausages and dry-cured bacon of Croatian and Slovenian origin, refers primarily to the OTA. The highest levels of OTA was determined in prosciutto, about even 2-3 times higher than the maximum recommended level of 1 µg/kg prescribed in certain EU countries. AFB₁ levels were not statistically significantly higher than the limit of

detection of used analytical method and indicate a negligible contamination of the products with this mycotoxin.

Acknowledgements

The authors are grateful for financial support from Ministry of Science, Education and Sports of the Republic of Croatia and Ministry of Higher Education, Science and Technology of the Republic of Slovenia (Project: „Improving the quality and safety of Croatian and Slovenian traditional meat products (TMP)“).

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Delivered: 17.11.2014. Accepted: 19.11.2014.