



SHORT COMMUNICATION

A survey on the occurrence of ochratoxin A in feeds and sera collected in conventional and organic poultry farms in Northern Italy

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ABSTRACT

A survey has been conducted on conventional and organic poultry farms located in northern Italy in order to investigate the occurrence of ochratoxin A (OTA) in feeds and sera in 2006. Ten poultry farms were monitored by taking 20 samples of feed and 94 samples of blood. OTA was assessed through immunoaffinity column purification and HPLC analysis. For in-house validation, recovery experiments, carried out on the spiked samples in the range of 1.0-10.0 $\mu\text{g OTA kg}^{-1}$ and 0.3-3.0 ng OTA ml^{-1} for the feed and serum samples, respectively, led to overall recovery averages of 80.6% (RDS=7.3%, n=9) and 83.3% (RDS=3.1%, n=9), respectively. All the feed samples were contaminated by OTA with values ranging from 0.04 to 6.50 $\mu\text{g kg}^{-1}$. Fiftythree percent of the sera samples were positive, with values ranging from 0.003-0.165 ng ml^{-1} . None of the feed samples was above the limits set by the European Union on OTA contamination in poultry feeds. No statistically significant differences in OTA contamination of feed or sera were observed either between the organic vs conventional group or between the laying hens vs broiler group.

Key words: Poultry, Feed, Serum, Ochratoxin A, HPLC.

RIASSUNTO

INCIDENZA DELLA CONTAMINAZIONE DA OCRATOSSINA A IN MANGIMI E SIERI PROVENIENTI DA ALLEVAMENTI AVICOLI CONVENZIONALI E BIOLOGICI DEL PIEMONTE (ITALIA NORD-OCCIDENTALE)

Nel corso del 2006 è stata condotta in Piemonte un'indagine in aziende avicole convenzionali e biologiche per valutare l'incidenza della contaminazione da ocratoxina A (OTA) in mangimi e sieri. Sono state

oggetto di studio un totale di dieci aziende avicole (6 convenzionali e 4 biologiche). Sono stati raccolti globalmente 20 campioni di mangime completo e 94 campioni di siero di galline ovaiole o di polli da carne. La presenza di OTA nei campioni è stata valutata mediante analisi in HPLC, previa purificazione del campione su colonnina ad immunoaffinità. Per la validazione del metodo sono stati effettuati degli esperimenti di recupero mediante contaminazione artificiale delle matrici in esame con 1,0-10,0 μg OTA kg^{-1} per i campioni di mangime e 0,3-3,0 ng OTA ml^{-1} per i campioni di siero. Le percentuali di recupero ottenute sono risultate pari a 80,6% (RDS=7,3%, n=9) per i campioni di mangime e a 83,3% (RDS=3,1%, n=9) per i campioni di siero.

Tutti i campioni di mangimi sono risultati contaminati da OTA, con valori compresi nell'intervallo 0,04-6,50 μg kg^{-1} . Il 53% dei sieri analizzati è risultato positivo alla contaminazione da OTA, con valori compresi tra 0,003 e 0,165 ng ml^{-1} .

In nessun caso sono stati rilevati livelli di contaminazione di OTA dei mangimi superiori ai limiti fissati dall'Unione Europea per gli alimenti completi destinati alle specie avicole. Sia per i mangimi che per i sieri, non sono state osservate differenze statisticamente significative né tra campioni provenienti da allevamenti convenzionali o biologici né tra campioni provenienti dalla filiera uova o dalla filiera carne.

Parole chiave: Avicoli, Mangime, Siero, Ocratossina A, HPLC.

Introduction

There has been a consistent increase in the number of organic farms in the EU over the last few years. Several surveys have found that consumers are willing to pay extra for organic products (Beharrell and MacFie, 1999; Hermansen, 2003), since a large proportion of consumers relate organic production to healthier food, less harm to the environment and better animal welfare (Sundrum, 2001). However, scientific evidence in support of the perception that environmentally friendly organic agriculture techniques are synonymous with the production of safe food, is scarce (Magkos *et al.*, 2006). Very few investigations have been carried out on the safety of organic food and even fewer on the safety of organic products of animal origin.

Since effective synthetic fungicides are not allowed in organic production, it is logical to assert that organic foods may be more susceptible to fungal contamination. From this, some authors have supposed that mycotoxin contamination could occur more frequently in organic feedstuff rather than in conventional ones (Kouba, 2002). However, according to the very few investigations on

this subject and to the controversial results, a FAO's report in 2000 concluded that there is no evidence to show that the risk of mycotoxin contamination in organic systems is higher than in conventional systems (FAO, 2000).

Ochratoxin A (OTA) is an ubiquitous mycotoxin produced by fungi of the *Penicillium* and *Aspergillus* genus. OTA is nephrotoxic and it is suspected of being the main etiological agent responsible for human Balkan endemic nephropathy (BEN) and associated urinary tract tumors (Pfohl-Leszkowicz and Manderville, 2007). International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B).

As OTA is highly stable during the usual feed storage and feed preparation procedures, it may endanger humans as the final consumers of contaminated food of plant and animal origin. Human intake and absorption of OTA has been confirmed through the detection of OTA residues in human blood serum, milk and kidney (Bauer and Gareis, 1987; Breitholtz *et al.*, 1991). Plant-based food, rather than food of animal origin, is considered to be the most important OTA source in the human diet. However, among

food of animal origin, it has been shown that the carry-over of OTA from feed to animal products can occur in swine and poultry (Micco *et al.*, 1987). Swine and poultry diets are based on cereals and cereal by-products up to 50-60% on a dry matter basis, and these raw materials are the preferred substrate for *Penicillium* and *Aspergillus* growth (Petzinger and Weindenbach, 2002).

In Italy, poultry production is the largest production, in terms of livestock products. The export - import balance is mostly positive. The Italian poultry population is estimated to be around 170,000,000 birds per year. Around 74% of the animals is reared in northern Italy and around 10% in Piedmont (north-west Italy) (ISTAT, 2000).

The present study is aimed at investigating the occurrence of OTA contamination in feeds and sera from (i) chicken broilers and laying hens (ii) organic and conventional farms located in north-west Italy.

Material and methods

Chemicals

An OTA stock solution (0.5 mg/ml) was purchased from Supelco (St. Louis, MO, USA). OTA standard solutions were prepared for HPLC calibrations or spiking purposes by dissolving amounts of the stock solution previously evaporated to dryness, in the mobile phase or in methanol, respectively. Acetonitrile and methanol (HPLC grade) were purchased from Merck (Whitehouse Station, NJ, USA). OchraTest™ immunoaffinity columns were supplied by Vicam L.P. (Watertown, MA, USA).

Feed and blood sampling

Ten poultry farms (5 laying hens farms, 3 of which were conventional and 2 organic, and 5 broiler farms, 3 of which were conventional and 2 organic) were monitored in 2006, by taking feed and blood samples.

Each farm was sampled one time. The farms were all in Piedmont, in north west Italy. About 1 kg of feed was sampled from each farm by the National Veterinary Service, according to EC regulation 2006/401/EC, for a total of 20 samples. Complete feeds from conventional farms were a standard formulation of 55-65% corn and 25-35% soybean meal. Complete feeds from organic farms were a formulation of 40-50% corn, 15-25% barley, 15-25% soybean meal, 5-8% pea, 0-3% alfalfa dehydrated meal and 2-5% corn gluten meal. The samples were stored at 20 °C until OTA extraction was performed. Blood samples were collected from about ten animals per farm, for a total of 94 blood samples. A blood sample of at least 10 milliliters per animal was taken from the jugular vein and collected in individual vacuum tubes. The collected blood was kept at 5 °C for 24h to allow serum separation.

OTA extraction and purification

Feed. The feeds were extracted and purified according to Entwisle *et al.* (1997), with some modifications. The samples were freeze dried in a lyophilizer (5Pascal, Trezzano sul Naviglio, Italy) for 2 d. The vacuum level was 1×10^{-1} mBa, and the temperature was -40 °C. About 500 g of each sample was finely ground in a Cyclotec 1093 mill (Foss, Hillerød, Denmark), equipped with a 1 mm mesh screen, and then homogenized. A 25 g sample was extracted with 100 ml acetonitrile-water (60:40, v/v) by blending with Polytron (Kinematica, Littau, Switzerland) at high speed for 3 minutes, on ice. The extract was filtered through filter paper and 4-ml filtrate was collected and mixed with 40 ml PBS. A 20-ml volume of the filtrate was purified on a OchraTest (Vicam L.P., Watertown, MA, USA) immunoaffinity column. The eluted extract was evaporated in a Speed Vac Concentrator (Savant Technologies, Rockville, MD, USA) at 45 °C and

reconstituted with 500 µl of the HPLC mobile phase. Sample extracts were stored at -20 °C until HPLC analysis. Recovery experiments were performed in triplicate by spiking blank feed samples with OTA at the levels of 1, 5 and 10 µg kg⁻¹.

Serum. The determination of OTA in serum was carried out according to Zimmerli and Dick (1996), with some modifications. A 10 ml serum sample was extracted with 10 ml of 3.4% H₃PO₄, 12% NaCl, pH<1 and 5 ml CH₃Cl, three times. The extracted sample was purified on an OchraTest (Vicom L.P., Watertown, MA, USA) immunoaffinity column. The eluted extract was evaporated in a Speed Vac Concentrator (Savant Technologies, Rockville, MD, USA) at 45 °C and reconstituted with 500 µl of the HPLC mobile phase. Recovery experiments were performed in triplicate by spiking blank serum samples with OTA at levels of 0.3, 1 and 3 ng ml⁻¹.

HPLC determination of OTA

The HPLC apparatus consisted of a Dionex P680 pump (Dionex, Sunnyvale, CA, USA) equipped with a Rheodyne Model 7725i injection valve (Rheodyne, Rohnert Park, CA, USA), a Dionex RF-2000 fluorimetric detector ($\lambda_{\text{ex}}=333$, $\lambda_{\text{em}}=460$), a Dionex thermostatted column compartment TCC-100, and a Chromeleon® 6 data handling system (Dionex, Sunnyvale, CA, USA). The analytical column was a Supelcosil LC-18 column (15 cm X 4.6 mm, 5 µm particles) (Supelco, Bellefonte, PA, USA), preceded by a Supelguard (Supelco, Bellefonte, PA, USA) guard column. Twenty µl of reconstituted feed and serum extract was injected into the chromatographic system by a full loop injection system. The system was run isocratically with a mobile phase containing acetonitrile-water-acetic acid (49.5:49.5:1, v/v), at a flow rate of 1 ml/min (Visconti *et al.*, 2000). The sample extracts that contained OTA were analyzed in duplicate with

a direct spiking of the second aliquot. This was performed by adding an amount of an OTA stock solution to the aliquot. The chromatograms of the unspiked and spiked sample extracts were then compared. The detected OTA was quantified against an external standard, after adjustments through regression lines.

Statistics

Summary statistics (mean and standard deviation) were computed for each dependent variable. The OTA contamination values were logN transformed to obtain log-normal distributed data. The effects of breeding system (organic *vs* conventional) and type of production (chicken broiler *vs* laying hen) on the feed and serum OTA concentrations were determined by ANOVA on the transformed data, at a 5% probability level. Linear correlation between the square mean concentration of OTA in the feeds and sera of each farm was determined. All the statistical computations were performed using SPSS 12.0 Windows software (SPSS, Inc., Chicago, IL, USA).

Results

Recoveries, which proved to be convenient for both the feed and serum samples, are shown in Tables 1. For the feed (Table 1), the overall average recovery within the 1-10 µg kg⁻¹ spiking range was 80.6% and the average coefficient of variation (RDS) was 7.3%. For the serum, within the 0.3-3 ng ml⁻¹ spiking range, the overall average recovery was 83.3% and the average coefficient of variation (RDS) was 3.1%. The detection limit was 0.001 ng ml⁻¹, based on a signal-to-noise ratio of 3:1. The standard curve was linear ($r^2=0.9986$). Figures 1 and 2 show typical chromatograms of naturally contaminated samples of the feed and serum, respectively.

Table 1. Recoveries of the methods used for OTA determination in the poultry feed and serum.

	Spiking level ¹	Recovery \pm SD (%)	RSD (%)
Feed	1.0	75.0 \pm 7.1	9.4
	5.0	86.7 \pm 4.9	5.7
	10	80.0 \pm 14.1	17.7
Mean of means		80.6 \pm 5.8	7.3
Serum	0.3	81.3 \pm 1.8	2.2
	1	82.5 \pm 4.3	5.2
	3	86.3 \pm 1.8	2.1
Mean of means		83.3 \pm 2.6	3.1

SD=Standard Deviation (n=3 replicates).

RSD=relative standard deviation.

¹ For feed: $\mu\text{g kg}^{-1}$; for serum: ng ml^{-1}

Table 2 shows the overall occurrence of OTA in the feed samples. All 20 of the samples that were analyzed, were contaminated (100%) by OTA, at levels ranging from 0.04 to 6.50 $\mu\text{g kg}^{-1}$. All samples from organic farms contained OTA at levels ranging from 0.04 to 6.50 $\mu\text{g kg}^{-1}$. One hundred percent of the samples were contaminated in conventional

farms, at levels ranging from 0.09 to 4.08 $\mu\text{g kg}^{-1}$. As far as the type of production is concerned, 100% of the samples from laying hen farms were contaminated, with values from 0.04 to 6.50 $\mu\text{g kg}^{-1}$, similarly, in samples from broiler farms, 100% were found to be positive, with values from 0.003 to 3.31 $\mu\text{g kg}^{-1}$. No statistically significant differences

Table 2. Occurrence of OTA in the feed samples, according to the production type and to the farming type.

		Number of samples	Number of positives	% positive	Mean ($\mu\text{g kg}^{-1}$)	Square mean ($\mu\text{g kg}^{-1}$)	Range ($\mu\text{g kg}^{-1}$)
Conventional	Laying hens	6	6	100	0.99 \pm 1.52	0.48	0.13 - 4.08
	Broilers	6	6	100	0.91 \pm 1.27	0.19	0.09 - 3.31
	Total	12	12	100	1.76 \pm 3.17	0.40	0.09 - 4.08
Organic	Laying hens	6	6	100	3.31 \pm 2.62	1.31	0.04 - 6.50
	Broilers	2	2	100	0.71 \pm 0.93	0.26	0.05 - 1.37
	Total	8	8	100	2.66 \pm 2.54	2.55	0.04 - 6.50
	Total	20	20	100	1.64 \pm 2.04	0.46	0.04 - 6.50

Figure 1. Chromatogram of a feed sample naturally contaminated with 6.50 μg OTA kg^{-1} .

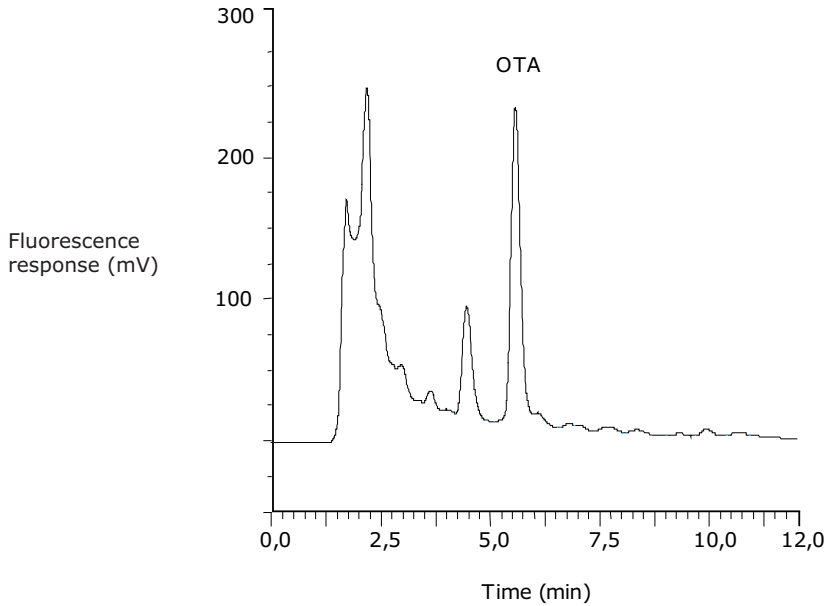


Figure 2. Chromatogram of a serum sample naturally contaminated with 0.165 ng OTA ml^{-1} .

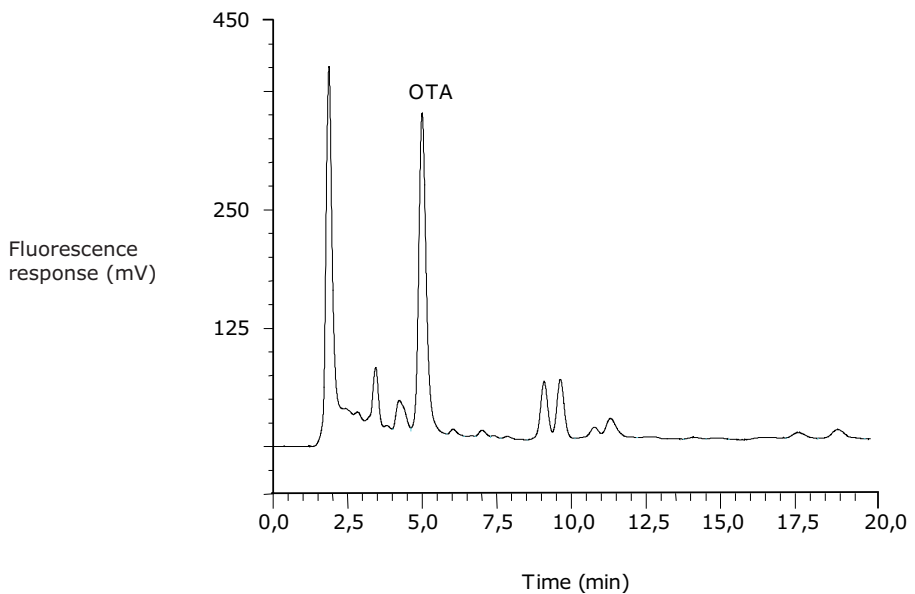


Table 3. Occurrence of OTA in the serum samples, according to the production type and to the farming type.

		Number of samples	Number of positives	% positive	Mean (ng ml ⁻¹)	Square mean (ng ml ⁻¹)	Range (ng ml ⁻¹)
Conventional	Laying hens	25	13	52	0.015 ± 0.020	0.005	0.006 - 0.082
	Broilers	30	13	43	0.012 ± 0.026	0.003	0.006 - 0.131
	Total	55	26	47	0.013 ± 0.024	0.004	0.006 - 0.131
Organic	Laying hens	26	19	73	0.030 ± 0.040	0.012	0.010 - 0.165
	Broilers	13	5	38	0.004 ± 0.004	0.002	0.003 - 0.014
	Total	39	24	61	0.022 ± 0.032	0.007	0.003 - 0.165
Total		94	50	53	0.020 ± 0.030	0.005	0.003 - 0.165

in OTA contamination were observed neither between the organic *vs* conventional group nor between the laying hen *vs* broiler group. The overall square mean value of OTA contamination found in feeds from organic farms was however numerically higher than that observed in conventional farms.

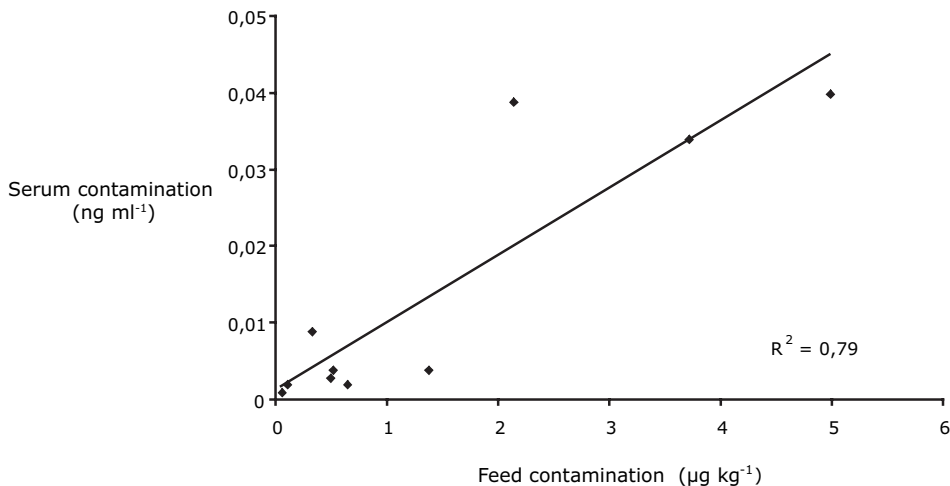
Table 3 shows the results of OTA detection in serum. OTA was detected in 50 out of the 94 serum samples that were analyzed (53%), at levels ranging from 0.003-0.165 ng ml⁻¹. The percentage of positive samples in organic farms was 61%, at levels ranging from 0.003 to 0.165 ng ml⁻¹. Forty seven percent of the samples in conventional farms were contaminated, at levels ranging from 0.006 to 0.131 ng ml⁻¹. The observed values did not differ significantly for the organic or conventional farms.

When regressing the OTA contamination of sera versus the OTA contamination of the corresponding feeds (Figure 3), a good linear correlation ($R^2=0.79$) was found and the carry-over rate resulted to be around 1%.

Discussion

None of the feed samples was above the limits set in EC recommendation 2006/576/CE on OTA contamination in poultry feed (100 µg OTA kg⁻¹, for feed at 12% moisture). No recent references have been found in literature concerning surveys of OTA levels in feedstuffs in Italy. As far as other countries are concerned, Jaimez *et al.* (2004) detected similar OTA findings on different feed and raw materials samples in Spain, with 33% positive samples at contamination levels ranging from 0.42 to 6.19 µg kg⁻¹. In particular, the highest values were observed in corn, corn gluten meal, cotton seed and palm kernel, while the mean values of OTA contamination for laying hens and broiler feeds were 0.50 and 0.52 µg kg⁻¹, respectively. Domijian *et al.* (2005) in Croatia, found OTA in corn in 39% of the analyzed samples, with a mean value of 1.47±0.38 µg kg⁻¹. In a survey conducted in the UK on ingredients for animal feeding stuffs (Scudamore *et al.*,

Figure 3. OTA contamination of sera vs OTA contamination of the corresponding feed. Each value of serum contamination in the graphic correspond to the mean value of serum contamination for each farm.



1998), only 5% of the analyzed maize gluten samples were found positive, with contaminations of up to 2 µg kg⁻¹.

Only a few data are available on the level of OTA contamination of sera from livestock animals. Some data for pig sera were reported in the surveys by Marquardt *et al.* (1988) and Curtui *et al.* (2001). In the study by Marquardt *et al.* (1988), the contaminated pig sera samples showed values of up to 200 ng ml⁻¹. Curtui *et al.* (2001) found 94% of the samples positive, ranging from 0.1 to 13.4 ng ml⁻¹. The highest value (0.165 ng ml⁻¹) found during the present survey was very much below the highest values described for pig sera in the reported studies. This difference could be explained by the toxicokinetics of OTA, which differs from animal to animal. OTA binding to the serum albumin and recycling in the bile and kidneys contribute to its general long half-life in animals. However, poultry species appear to eliminate OTA faster than mammals do and this leads to a lower OTA accumulation in the blood (Leeson *et al.*, 1995).

Conclusions

In conclusion, OTA contamination is widespread both in feed and sera samples collected in conventional and organic poultry farms in Piedmont, at low contamination levels. The OTA contamination levels found in feeds were very much below the limit (100 µg OTA kg⁻¹) recommended by the European Commission (2006/576/EC) for complete and complementary poultry feedstuffs. No differences in OTA contamination were found between conventional and organic farms.

All the authors contributed equally to the work described in this paper.

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