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# Determination of Ochratoxin A in Poultry Feeds Available in Rabat area (Morocco) by High Performance Liquid Chromatography

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## Abstract
Sixty two (n=62) samples of poultry feeds collected from farms in three different cities of the Rabat area (region) in Morocco were surveyed for the presence of ochratoxin A (OTA). Samples were extracted with an-in house validated method. Influence of several extraction solvents was studied for the method optimization. OTA was then identified and quantified with liquid chromatography (LC) coupled to fluorescence detection. Results showed that methanol gives the best average recovery (81.0±1.5 %). The limit of quantification (LOQ) was 0.15 ng/g. The proposed method was successfully applied to the analysis of samples. OTA was present in 19 (30.6%) out of total analyzed samples. OTA levels varied between 0.24 and 26.8 ng/g. The average levels of OTA were 11.3, 0.8 and 9.4 ng/g in samples from farms in Rabat, Salé and Témara, respectively. No sample exceeded the maximum limit (100 ng/g) recommended by the European legislation for OTA in poultry feeds.

*Keywords: Contamination, Feed, Ochratoxin A, Poultry, HPLC, Morocco*

## 1. Introduction

In Morocco, poultry sector plays an important role in the national economy. According to a report from the Entrepreneurship Observatory (OE), the sector torn between a traditional farming activity and intensive modern production. It generates over 23.2 billions of Moroccan dirhams MDH (2.8 billion US dollars) and has made a direct investment of 1 billion US dollars in 2010. Nowadays, the sector provides 360 000 direct and indirect jobs, including marketing channels and distribution [1].

Given its rapid development capabilities, the poultry sector is a competitive alternative to meet the nutritional needs of a growing population, marked by high urbanization and low purchasing power. In 1999, the Moroccan Ministry of Agriculture (MMA) adopted a law (n° 49-99) to protect the health of poultry farms [2]. In 2011, poultry meat production has reached 516 955 tons, but the sector remains dependent on the feeds import. Laying hens receive balanced food enriched with additional vitamins, minerals and trace elements, guarding against nutritional deficiencies. However, the sector suffers from a lack of synchronization between the various links in the chain; including poultry feed safety. Indeed, little information is now available in Morocco on the conformity of poultry feeds to international standards.

Various diseases of unknown etiology in poultry have been reported being associated with ingestion of contaminated feeds with mycotoxins [3]. Subclinical immunosuppression in chickens can be caused by pathogens such as chicken infectious anemia virus, infectious bursal disease virus, reovirus, and some retroviruses (e.g., reticuloendotheliosis virus). However, mycotoxins and stress, often caused by poor

management practices, can also cause immunosuppression in chickens [4]. The most known mycotoxins in poultry food chain are aflatoxins (AF) and ochratoxin A (OTA). Exposure to mycotoxins may result in acute, overt disease, or as is usually the case, chronic, insidious exposure that impairs poultry productivity. The severity of any of these effects in poultry production systems will depend on the level of mycotoxin present in the feed supply chain, the duration of exposure, the physiological status of the animal and other environmental and disease factors that impact on the uptake, biotransformation, deposition and excretion of theses toxins [3]. These contaminants of agricultural commodities have attracted worldwide attention because of the significant losses associated with their effects on human health and livestock [5]. Animal feed is the first link of food chain; therefore the risk of contaminant carryover from contaminated feeds to animal tissues and biological fluids, and eventually to products intended for human consumption (meat, eggs) is a matter of concern [6].

The International Agency for Research on Cancer has classified OTA in group 2B as a possible carcinogenic compound to humans [7]. OTA is a natural contaminant of farm animal feeds throughout the world, posing a potential threat to animal production. Farm livestock ochratoxicosis episodes have occurred in some countries [8]. The first spontaneous toxic avian nephropathy associated with OTA exposure was reported in Denmark by Elling *et al*. [9], which detected microscopical renal changes in four out of 14 kidneys. Later in the United States, Hamilton *et al*. [10] reported five independent episodes of ochratoxicosis in turkeys, in laying hens and in broiler chickens. In poultry, OTA is recognized to increase the susceptibility and aggravate the clinicopatological picture in case of coccidiosis, salmonelosis and colibacillosis [8]. The common clinical symptoms observed in ochratoxicosis are retardation of growth, dullness, huddling, decrease appetite, reduced feed intake, weakness and diarrhea; OTA also affects egg production, fertility and hatchability [11].

To prevent sanitary and economic negative impacts in poultry, the European Commission Recommendation 2006/576/EC, suggests that the maximum residue level (MRL) of OTA in poultry feeds should be set at 100 ng/g [12]. However, it was reported that feeding broiler chickens a diet contaminated with the MRL had an overall immunosuppressant effect, with reduction in the thymus weight and of the total serum protein, albumin, alpha, beta and gamma globulins concentration [13].
Due to its toxicity, OTA presence is increasingly regulated worldwide, however, up until now, no regulation limits are in force in Morocco [14]. Assessing the mycotoxin risk in animal feed remains a difficult and challenging problem. Commercial feedstuffs are an important component in modern animal husbandry, but few studies on mycotoxins in poultry feeds are available in the country. In a previous study, poultry feeds from Morocco were found contaminated by aflatoxins [15]. However, the presence of OTA in such feeds has never been investigated. The purposes of this investigation were to validate an internal method for the determination of OTA in poultry feeds by liquid chromatography coupled to the fluorescence detection, the optimization of the extraction procedure relating to solvent use was also studied; and to evaluate for the first time the presence of OTA in samples of poultry feeds available in Morocco.

## 2. Materials and Methods

*2.1 Chemical and Reagents*
OTA crystalline material was purchased from Sigma (St. Louis, MO). A stock standard solution of OTA at 500 ng/mL in methanol was prepared and kept wrapped in aluminum foil at -20 °C. OTA working solutions (20 and 50 ng/mL) were prepared by dilution in the same solvent and stored in glass-stoppered tubes at -20 °C. High-performance liquid chromatography (HPLC) grade methanol and acetonitrile were supplied by Merck (Darmstadt, Germany). Acetic acid was obtained from Scharlau (Barcelona, Spain). Deionized water (0.125 lS) was obtained using a Milli-Q water purification system.

*2.2 Sampling*
Sixty two (62) samples of poultry feed, composed by energy giving materials (corn derivatives products), protein and minerals sources, were collected from different poultry farms located in Rabat (n=18), Témara (n=23) and Salé (n=21) in Morocco during 2013 and 2014. Samples were stored in plastic bags, taken to the Laboratory. At the arrival, all collected samples were milled and stored in a dark and dry place until analysis of OTA.

*2.3 Sample fortification*
Recoveries of the extraction method were determined by sample fortification. 2.5 g of milled free sample of poultry feed were fortified 1 h before extraction with a solution of OTA in methanol at 0.05 µg/mL. Samples were artificially contaminated in triplicates with this solution in order to obtain the desired concentration. After the fortification and homogenization, samples were analyzed as described below.

*2.4 OTA extraction procedure*
The method used for OTA extraction from poultry feed was developed in the laboratory by using some modifications of the method described by Mol *et al*. [16]. Briefly 2.5g of poultry feed samples were extracted with 10 mL of methanol. The mixture was shaken end-over-end for 1h and centrifuged at 4500 rpm for 10 min. The supernatant evaporated to dryness using a Stuart Rotavapor RE-300, and then re-dissolved in 1 mL of the mobile phase (acetonitrile/water/acetic acid, 50/49/1 v/v/v). The final solution was filtered using a Millipore cellulose filter (0.22 µm of porosity) before the injection into the LC system for analysis.

*2.5 Liquid chromatography-fluorescence detection*
LC analysis of OTA was performed using a Perkin Elmer (USA) LC system equipped with a micro pump series 200, an autosampler series 200 and a fluorescence detector series 200. An LC column Phenomenex (Res Elut C18, 250 mm x 4.5 mm i.d., 5µm) was used with a mobile phase of acetonitrile /water/acetic acid (50:49:1 v/v/v) at a flow rate of 0.4 mL/min. Detection of OTA was carried out using 334 and 464 nm as wavelengths for excitation and emission, respectively.

*2.6 OTA confirmation procedure*
The confirmation of OTA identity in positive samples was cheeked by the formation of a methyl OTA ester after methylation of the toxin [17]. For this, an aliquot of the final extract of the sample (200 µL) was diluted with methanol (2.5 mL) and concentrated HCl (0.1 mL). The solution was left standing overnight at room temperature in dark conditions. The methanol was then evaporated, and the residue was taken up in 200 µL methanol-formic acid 0.1 M (70:30, v/v) and injected into the LC apparatus under the same chromatographic conditions described above.

## 3. Results and discussion

*3.1 Method performance*
In order to carry out a study on the presence of OTA in poultry feed samples, a validation of an internal method was first investigated. Modifications were made to avoid using high amounts of solvents for extraction of the OTA with a small quantity of feed sample (2.5 g). The selection of a suitable solvent is the most challenge in the optimization of a method for OTA analysis. Several extraction solutions were tried with different solvents (alone or mixed): acetonitrile/water/formic acid (89/10/1 v/v/v), methanol/water/formic acid (89/10/1 v/v/v), water/acetonitrile (84/16 v/v), acetonitrile/water/acetic acid (79/20/1 v/v/v), methanol (100%) and acetonitrile (100%). The influence of different solvent mixtures on OTA recoveries was studied in order to achieve the most efficient extraction. Recoveries of OTA were calculated for each solvent used by spiking OTA-free samples. As shown in Table 1, all solvents used were able to extract OTA with different capabilities. After several assays, methanol (100%) was selected for the extraction of OTA due to the best recoveries obtained. Indeed, this solvent gives an average recovery of about 81.0±1.5 % at a fortification level of 10 ng/g. Several parameters (linearity, reproducibility, repeatability, limit of detection and limit of quantification) were cheeked to study the performance of the method.
All the objectives for linearity validation have been matched. The coefficient of correlation obtained ($r^2$ = 0.988) indicated a correct calibration curve (up to 25 ng/g). At the fortification level, an inter-day variation, for 3 days, between 7% and 12% an intraday variation, n = 3, between 4 % and 10% were obtained. The estimated LOD, S/N 3:1, and LOQ, S/N 10:1, were calculated to be 0.05 and 0.15 ng/g, respectively.

*3.2 OTA in poultry feed samples*
The occurrence of OTA in analyzed samples is represented in Table 2. As can be seen, OTA was present in 19 (30.6%) out of total 62 total analyzed samples. Levels of OTA in positive samples ranged from 0.24 to 26.8 ng/g. According to data obtained, all positive samples were below the maximum level (100 ng/g) of OTA

recommended by EU regulations in poultry feeds. Figure (1B) shows a chromatogram of a naturally contaminated sample of poultry feeds.
The highest frequency of positive samples (33.3 %) was found in samples from Rabat and Salé farms. While the highest OTA value (26.8 ng/g) was found in samples from Rabat farms. As far as we know, this is the first report on the contamination of poultry feeds available in Morocco by OTA.

**Table 1:** Results of OTA recovery assays using different mixture of solvents.

| **Solvents** | **Mean recovery ** (%) of OTA** |
|---|---|
| Acetonitrile /water/formic acid (89/10/1) | 41.6±0.4 |
| Methanol/water/formic acid (89/10/1) | 48.5±1.7 |
| Water /Acetonitrile (84/16) | 71.3±1.4 |
| Acetonitrile /water /acetic acid (79/20/1) | 61.6±2.4 |
| Methanol (100%) * | 81.0±1.5 |
| Acetonitrile (100%) | 8.6±3.2 |

* This solvent was selected for better recovery and minor RSD.
** The result for every solvent mixture is a mean of three fortified samples on two representative samples of poultry feeds.

**Table 2** : Occurrence of OTA in analyzed samples.

| Origin | Number of samples | Ochratoxin A levels | | |
|---|---|---|---|---|
| | | Positive samples (Frequency) % | Mean value (ng/g) | Maximum level (ng/g) |
| *Rabat* | 18 | 6 (33.3%) | 11.3±2.5 | 26.8 |
| *Salé* | 21 | 7 (33.3%) | 0.8±1.5 | 1.8 |
| *Témara* | 23 | 6 (26.1%) | 9.4±0.5 | 18.0 |
| *Total* | 62 | 19 (30.6%) | 7.1±2.4 | 26.8 |

The contamination of poultry feeds and feed ingredients by fungi is worldwide documented, and mycotoxin production and occurrence in poultry feeds have been studied extensively, as well as the consequences for avian health and well-being [18]. According to Lozada [19], the presence of moulds and mycotoxins in poultry feeds results in the raw materials used in their production. Rosa *et al*. [20] reported that *Aspergillus flavus* and *Penicillium citrinum* were the most prevalent species isolated from poultry feeds in Brazil. Authors showed that there was a high percentage of potential OTA producers (46%) among isolates. Labuda and Tančinová [21] surveyed the distribution and the toxinogenity of fungi isolated from Slovakian poultry feeds mixtures, and reported that OTA was detected in two *Penicillium verrucosum* and seven *Aspergillus*

*ochraceus* isolates, while none out of *A. niger* isolates was able to produce OTA. Fraga *et al*. [22] monitored the mycobiota counts at different stages of poultry feed processing and found high levels of OTA producers at all stages. Figueroa *et al*. [23] reported that the most frequently isolated genera of moulds from concentrated poultry feed were *Aspergillus* (36%) and *Penicillium* (20%), ochratoxigenic species were *Eurotium herbariorum*, *Aspergillus niger*, *Aspergillus ochraceus* and *Aspergillus glaucus*. Farmers are often tempted to incorporate mouldy grain into animal diets to reduce feed costs. However, this practice causes a risk for mycotoxin contamination, and alters nutrient content of the grain.

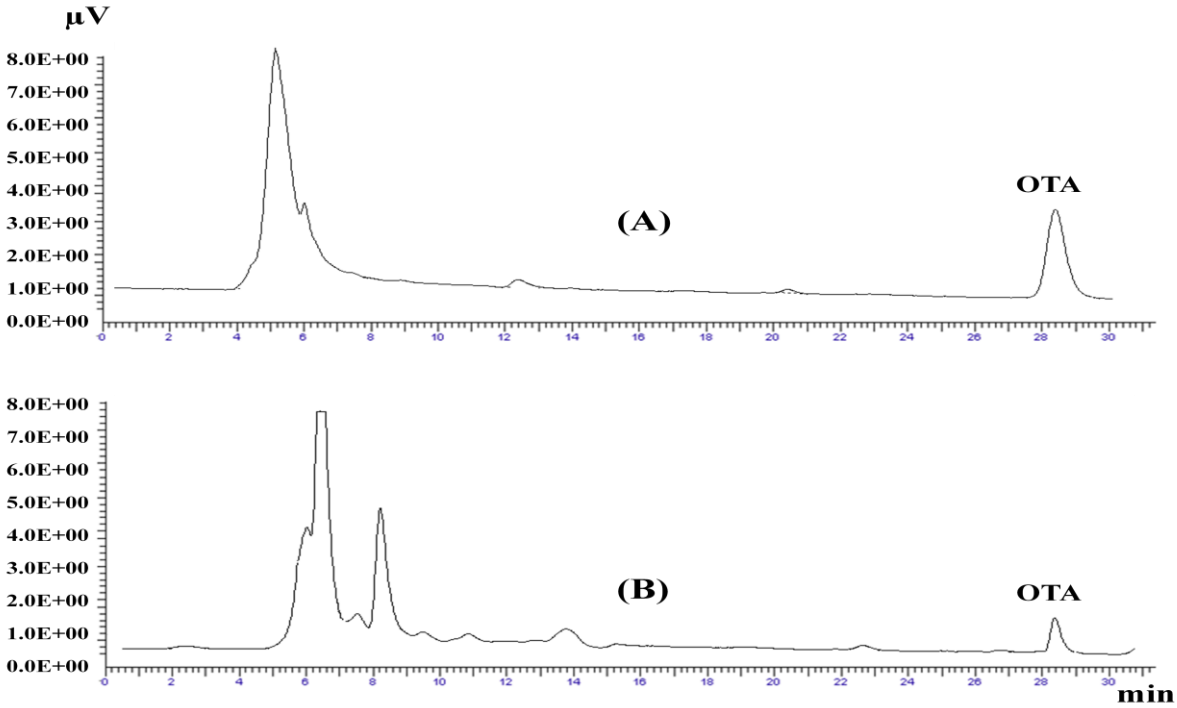


**Figure 1:** LC fluorescence chromatograms of : (A) an OTA standard solution at 20 ng/g; (B) an OTA contaminated poultry feed sample at 4.5 ng/g.

According to Bryden [3], changes in nutrient supply to the animal may alter its response to mycotoxin exposure and complicate diagnosis. For comparison, in a survey report conducted on the poultry feed ingredients in India, it was reported that 6 % of the samples were positive for OTA [24]. While in Venezuela, 98% of concentrated poultry feed samples were contaminated with OTA, levels ranged from 2.5 to 31.9 ng/g [23]. In a survey of conventional and organic poultry farms in northern Italy, Schiavone *et al*. [25] detected contamination with OTA in 100% of feed samples ranging from 0.04 to 6.5 ng/g and 53% of serum samples. In Portugal, it was reported that 12 sample feeds for laying hens (out of 186 total samples) were positive for OTA [26]. In Pakistan, OTA contamination of poultry feeds ranged between 10 and 112. 20 ng/g [27]. More recently, Sherazi *et al*. [28] showed that OTA occurred in complete poultry feed and samples of feed ingredients from Pakistan with mean OTA levels of 51 and 75 µg/kg, respectively.

## Conclusion

According to the results obtained from this study, it can be concluded that the analytical method reported herein could be applied to samples of poultry feeds for routine analysis of OTA in an official Moroccan laboratory in charge of feed safety. The application of this method to samples available in farms from Morocco showed that OTA was detected in 30.6 % of total samples. No positive samples were above the maximum limit (100 ng/g) recommended by EU countries in poultry feeds. Nowadays poultry feeds are not controlled for the presence of mycotoxins in the country, these results should spur Moroccan authorities to set a national monitoring program for mycotoxins analysis in animal feeds to improve the quality of imported feeds and reduce human exposure. It is also interesting to study the possible correlation between the presence of OTA in poultry feeds and its prevalence in sera of birds.

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