Co-occurrence of mycotoxins in poultry feed and feed-ingredients marketed in Morocco

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Abstract

This study investigated the contamination of poultry feed-ingredients and compound feed used in Morocco. ELISA analysis was performed on a total of 73 samples with corn (n = 59), soy (n = 8), and wheat (n=6). HPLC-MS/MS method was used for the quantification of Aflatoxin B1 (AFB1), Zearalenone (ZEA), Deoxynivalenol (DON), and fumonisin B1 (FUMB1) in corn (n = 10), soy (n = 5), wheat (n = 5), and compound feed (n = 18). Results of ELISA showed that the analyzed corn samples contained at least AFB1, DON, FUMB1 at the respective mean concentrations of 2.88, 3213, and 6241ug/kg. For soya and wheat, the respective mean concentrations were 26 and 45 ug/kg of FUMB1, meanwhile DON was only detected in wheat samples at the mean concentration of 2,80 ug/kg. The HPLC-MS/MS showed that corn samples were contaminated by AFB1, DON, FUMB1, ZEA with average concentrations of 0.35, 2523, 6542, and 41.9 ug/kg, respectively. Soybean samples were contaminated with DON, FUMB1, and ZEA at the mean concentrations of 6.6, 73.9, and 75.7 ug/kg, respectively. Wheat were contaminated by DON, FUMB1, and ZEA, at average concentrations of 1.99, 179, 4.01 ug/kg, respectively. AFB1, DON, FUMB1, and ZEA were detected in compound feed at average concentrations of 0.73, 3420, 4072 and 59 ug/kg, respectively. However, in all samples, mycotoxins concentrations did not exceed the maximum limits of the European Union standards and ochratoxin was not detected.

Key words: Aflatoxins, Zearalenone, Deoxynivalenol, OchratoxinA, Fumonisins, Corn, Soybean, Poultry compound feed

Co-occurrence de mycotoxines dans les aliments pour volailles et les ingrédients alimentaires commercialisés au Maroc

Résumé

L'objectif de notre étude est d'examiner la contamination des matières premières pour aliments de volailles et les aliments composés utilisés au Maroc. 73 échantillons ont été analysés par la méthode ELISA avec (n = 59) pour le maïs, le soja (n = 8) et le blé (n = 6). La méthode HPLC-MS/MS a été utilisée pour la quantification de l'aflatoxine B1 (AFB1), de la zéaralénone (ZEA), du désoxynivalénol (DON), de la fumonisine B1 (FUMB1) et de l'OchratoxinA (OTA) dans le maïs (n = 10), le soja (n = 5), le blé (n = 5) et les aliments composés fabriqués avec ces matières premières (n = 18). Les résultats de l'ELISA ont montré que les échantillons de maïs analysés contenaient au moins AFB1, DON, FUMB1 aux concentrations moyennes respectives de 2,88; 3213 et 6241 ug/kg. Pour le soja et le blé, les concentrations moyennes respectives étaient de 26 et 45 ug/kg de FUMB1, alors que le DON n'a été détecté que dans les échantillons de blé à la concentration moyenne de 2,80 ug/kg. La méthode HPLC-MS/MS a montré que les échantillons de maïs étaient contaminés par AFB1, DON, FUMB1, ZEA avec des concentrations moyennes de 0,35; 2523; 6542 et 41,9 ug/kg, respectivement. Les échantillons de soja ont été contaminés par DON, FUMB1 et ZEA respectivement aux concentrations moyennes de 6,6; 73,9 et 75,7 ug/kg. Le blé a été contaminé respectivement par, DON, FUMB1 et ZEA à des concentrations moyennes de 1,99; 179 et 4,01 ug/kg. AFB1, DON, FUMB1 et ZEA ont été détectés respectivement dans les aliments composés à des concentrations moyennes de 0,73; 3420; 4072 et 59 ug/kg. Cependant, dans tous les échantillons, les concentrations de mycotoxines n'ont pas dépassé les limites maximales des normes de l'Union européenne et l'ochratoxine n'est pas détectée dans l'ensemble des échantillons.

Mots clés: Aflatoxines, zéaralénone, déoxynivalénol, ochratoxine, fumonisines, maïs, soja, aliments composés pour volailles

INTRODUCTION

Mycotoxins are natural toxicants produced by various fungi, which have been associated with serious health problems in humans and animals. Although, it is well admitted that these natural contaminants are difficult to eradicate from foods and feeds, it is, nonetheless, possible to reduce substantially their incidence and the associated health risks by the implementation of appropriate management and monitoring systems. The most common mycotoxins in poultry feeds and their ingredients are aflatoxins (AFLs), fumonisinB1 (FUMB1), deoxynivalenol (DON), ochratoxinA (OTA) and zearalenone (ZEA) which have often been mainly associated with grains used as ingredients in feed formulations (Asam *et al*., 2017). In fact, due to their high impact on human health, as well as on domestic animal health and productivity, they have been regulated in many countries, promulgating more or less stringent maximum tolerable limits (MTLs) or guidance levels (Kovalsky *et al*., 2016).

The poultry sector is playing increased role in economy and availability of animal proteins for consumers in Morocco. During the last decade, the yearly egg production has increased from 3900 to 6600 billion eggs, representing a growth rate of 1.7 fold, with a concomitant increase in poultry meat production from 440,000 to 670,000 tons, representing an overall increase of about 1.5 fold (http://www.fisamaroc.org.ma/). However, Morocco relies almost entirely on the importation of feeds and feed-ingredients, which should be properly inspected at the boarders and scrutinized for contamination with mycotoxins, among other control measures. There is an increased concern of the potential risk associated with mycotoxin-contaminated animal feeds and their effect on animal productivity as well as the adverse effects on human health due to the carry-over phenomenon on to foods of animal origin.

Few studies, to our knowledge, have been carried out on the detection and quantification of mycotoxins in feeds produced and/or marketed in Morocco (Zinedine, 2010; Zinedine, 2007). Therefore, the aim of this study was to

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evaluate the occurrence of mycotoxins in imported feed-ingredients (corn, soya, and wheat) and in the compound poultry feed produced in Morocco.

MATERIAL AND METHODS

Chemicals and reagents

Mycotoxin standards of fumonisinB1 (FUMB1), deoxynivalenol (DON), ochratoxin (OTC), zearalenone (ZEA) and aflatoxin B1 (AFB1), were obtained from Sigma Aldrich (Saint Louis, USA) and stored under the storage conditions recommended by the manufacturer.

HPLC gradient grade organic solvents (ethanol and methanol, acetonitrile), acetic acid (p.a), and sodium chloride (89%) were purchased from VWR International (Center Valley, USA).

Sample collection and preparation

A total of 111 samples of poultry feed and feed-ingredients, consisting of compound feed (n=18), corn (n= 69), soya (n=13), and wheat (n=11) were analyzed for contamination levels with FUMB1, DON, ZEA, OTC and AFB1 (Table 1).Samples were collected from Moroccan feed factories during the period of April 2016 to November 2017and tested according to the procedures used in the official control of food stuff in force in the European Union (EU) (Richard *et al*., 2000; Commission Regulation N° 401, 2006). From each lot, sub-samples were taken with a probe and mixed together to make up a 1 kg composite sample, which was stored in the freezer at -20 °C until needed.

Mycotoxins determinations

Composite samples were withdrawn from the freezer to be prepared for ELISA and/or High-Pressure Liquid Chromatography coupled to Mass Spectroscopy (HPLC-MS/MS) analysis as appropriate (Table 1).

Table 1: Techniques and sampling for mycotoxin detection and quantification in feed-ingredients and compound feed for poultry in Morocco

Product	Analytical technique		
	ELISA	LC-MS/MS	ELISA and LC-MS/MS
Corn	59	10	10*
Soya	8	5	5*
Wheat	6	5	5*
Compound feed	-	18	
	73	38	

** Same samples analyzed with ELISA and LC-MS/MS*

- No samples tested with this technique

ELISA was performed with AgraQuant® mycotoxin kit (Romer Labs Diagnostic GmbH, Butzbach, Germany) according to the manufacturer's instructions. Prior to testing, a 20 g sub-sample were extracted with different solvent systems depending on the mycotoxin to be analyzed. DON was extracted with 100 ml distilled water, while the other mycotoxins, were extracted with 100 ml of aqueous methanol (70:30). The same solvent system with added sodium chloride (1 g/100 ml) was used to extract fumonisinB1. After screening, the absorbance was read in an ELISA reader (Chro Mate, Awareness Technology Inc., Palm city, FL, USA) at 450 nm. The limit of detection (LOD) for each mycotoxin was as follows: 1 ppb (1 ppb = 1 ng/ml) for AFs, 250 ppb for DON and FUMB1, 40 ppb for ZEA and 1.9 ppb for OTA.

HPLC-MS/MS was performed for simultaneous detection/quantification of mycotoxins in feed ingredient and compound feed samples by using Agilent 1290 HPLC coupled to applied Biosystems 5500 QTrap mass spectrometer(Agilent Technologies, Waldbronn, Germany).Sample preparation and analysis were performed as described previously (Varga *et al.*, 2013; Zachariasova *et al*, 2010). Briefly, after crushing composite samples (about 250 g, each) in a Moulinex™grinder for 1 to 2 min, 5 g were weighed and extracted for 90 min with 20 ml acetonitrile/water/acetic acid (79:20:1) solvent. The mixture was then allowed to settle at room temperature until separation of the solid and liquid phases (10-15 mn). An aliquot (crude extract) was taken from the liquid phase, transferred into a fresh clean vial, and diluted with an equal volume of acetonitrile/water/acetic acid (20:79:1); 5 ml from this dilution was subjected to HPLC-MS/MS analysis. The limit of detection (LOD) were calculated from the signal to nose ratios (S/N), (LOD=3XS/N).The LOD values for each toxin by this method were 0.3 ppb for AFs,0.46 for ZEA, 1 ppb for FUM, and 26ppb for DON, 0,3 ppb for OTA. In the case of compound feed, they were 0,3 ppb for OTA? 0.72 ppb for AFs, 0,64 for ZEA, 2,6 ppb for FUM B1, and 9,5ppb for DON (Varga *et al.*, 2013; Zachariasova *et al.*, 2010).

RESULTS AND DISCUSSION

Occurrence of mycotoxins in Poultry Feed-ingredients and Compound Feed

ELISA analysis was used to screen for the presence of mycotoxins in corn, soya, and wheat samples imported in Morocco from the USA, Brazil, and Argentina during the period of April 2016 to November 2017 for poultry feed formulations. Only 26 and 45 µg/kg of FUMB1 were detected in soya and wheat samples, respectively; and 2,80 µg/kg of DON, which was only detected in wheat samples. However, corn samples were found to be co-contaminated by various mycotoxins, the most important of which were AFB1, DON, and FUMB1 with the respective average levels of 2.88, 3213 and 6241 µg/kg.

HPLC-MS/MS analysis revealed that the analyzed samples contained more than 3 different mycotoxins. AFB1, DON, ZEA, and FUMB1 being the most frequently encountered. Multi-mycotoxin occurrence in foods and feeds is a common phenomenon, and an agricultural commodity was reported to contain typically between 7 and 75 different mycotoxins and derived metabolites, with an average of 30 (Kovalsky *et al*., 2016; Schwab *et al*., 2019). Figure 1 illustrates the percentage of contamination with ZEA, AFB1, DON and FUMB1 of all of the analyzed samples regardless of the matrix. This figure shows that the so-called *Fusarium* mycotoxins e.g., DON, ZEA, and FUMB1, were the most frequent, with FUM B1 being detected in most samples (99%), followed by DON (93%) and ZEA (34%), while AFB1 was the least frequently detected, as it occurred in only 28% of the samples. Frequent occurrence and co-occurrence of these mycotoxins in cereal products is well established due to the fact that a mould strain can produce more than one mycotoxin (Asam *et al*., 2017; Kovalsky *et al*., 2016; Kamala *et al*., 2015).

For compound feed, the results summarized in Table 2 show that the highest contamination levels with these mycotoxins were recorded for FUMB1 (4072 mg/kg), followed by DON (3420 mg/kg), and ZEA (59 mg/kg).

For corn, soya and wheat, the results of AFB1, DON, ZEA, and FUMB1 concentrations are presented in Table 2. Among the analyzed feed ingredients (corn, soya, and wheat), corn was the most contaminated with the four mycotoxins, while the lowest concentrations were recorded in wheat samples, with the exception of FUMB1 whose lowest concentrations were detected in soya samples. The concentrations of AFB1 were low in corn samples (0.35 mg/kg on average) and below the detectable limit in wheat and soya samples. Corn contamination with high levels of aflatoxins has been abundantly documented, especially in tropical and sub-tropical countries, which include Brazil and Argentina (Kamala *et al*., 2015; Kamala *et al*., 2018). The low levels recorded in our samples may be explained by the period of the study, which corresponds to the dry season in Morocco, as high humidity is an essential environmental factor for aflatoxigenic molds to grow and produce aflatoxins (Gizachew *et al*., 2019). This also suggests that the feed mills that provided the samples store their products under appropriate conditions of controlled temperature and humidity that prevent them from mold contamination, growth, and mycotoxin production. The exporting country may also account for such low levels detected, indicating the implementation, by the exporter, of good pre- and post-harvest practices to produce high quality crops. Infrequent contaminations with low aflatoxin levels of corn from Brazil, one of the main exporter to Morocco, corroborates the latter assumption (De Lourdes Mendes de Souza *et al*., 2013). Conversely, corn samples contained relatively high concentrations of fumonisins and DON in accordance with the reported widespread contamination of corn with these mycotoxins (Yazar *et al*., 2008; Placinta *et al*., 1999). A previous study on the contamination of Moroccan corn with FUMB1, OTA, and ZEA showed that 50% (10 out of 20 samples) of the analyzed samples were contaminated with at least two of these mycotoxins (FUMB1 plus either OTA or ZEA), and one sample contained all three of them (Zinedine *et al*., 2006). Consistent with our results, the latter study reported that fumonisins were the most frequently detected mycotoxins and at the highest levels (up to 5960 µg/kg), while only 3 out of the 20 analyzed samples contained ZEA at the low levels of 12, 14 and 17 µg/kg.

The effect of mycotoxins in poultry are very complex. Mycotoxins present simultaneously in feed they may have synergetic and additive effect so with low levels of mycotoxins in feed and with a long periods of exposition we will get the immune suppression effect and more susceptible to disease.

Mycotoxin levels in compound feed samples were higher than those in the feed ingredients separately (Table 2).This contrast with the expected reduction of these levels during processing or by the dilution effect that results from the mixture of different ingredients, each having a specific

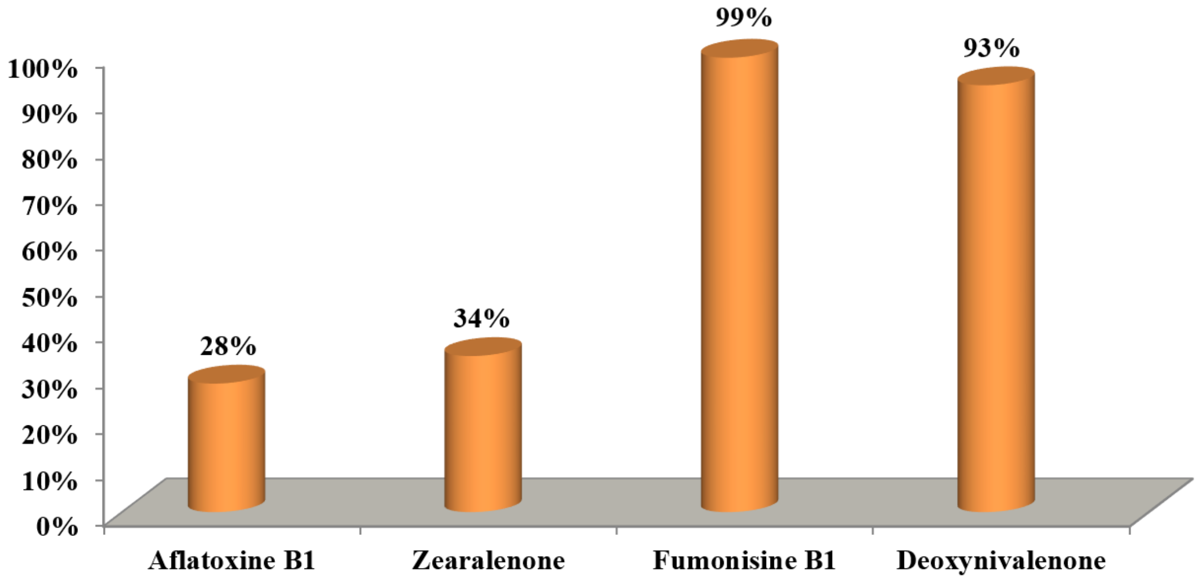

Figure 1: Prevalence of the main mycotoxins in a total of 111 samples including feed-ingredients and compound feed produced in Morocco during the period of April 2016 to November 2017

Table 2: Concentrations (mg/kg), determined by HPLC-MS/MS, of aflatoxin B1 (AFB1), zearalenone (ZEA), deoxynivalenol (DON), and fumonisins (FUMB1) in corn, soya and wheat used as poultry feed ingredients and compound feed in Morocco during the period from April 2016 to November 2017 (for all samples analyzed, the OTA is note detected)

Samples	Mycotoxins											
	AFB1			ZEA			DON			FUMB1		
	Mean*	Min**	Max***	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Corn	0.35	0.30	0.40	41.9	0.74	40.6	2523	87.0	7653	6542	1026	10850
Soya	ND	ND	ND	75.7	0.34	325.1	6.61	0.43	33.1	74.0	22.2	348
Wheat	ND	ND	ND	4.0	0.15	10.5	1.99	0.22	4.74	179	42.3	622
Compound feed	0.73	0.38	1.26	59.0	3.22	65.8	3420	87.1	681.2	4072	1530	9640

**Arithmetic mean; **Minimum value detected; ***Maximum value detected; ND: Not detected (<LOD)*

contamination profile. Compound feed was the most contaminated with AFB1 with concentrations varying between 0.38 to 1.26 µg/kg with an average of 0.73 µg /kg (Table 2). These results are comparable, although somewhat lower, to those reported previously in poultry feed marketed in Morocco showing that 66% of the analyzed samples were contaminated with AFB1 at concentrations in the range of 0.05 to 5.38 µg /kg and an average of 1.26 µg /kg (Zinedine *et al*., 2007). Higher contamination levels of aflatoxins were frequently reported in poultry feeds in various African and Asian countries where hot and humid climates prevail with mean values largely exceeding the EU regulatory MTL of 20 µg/kg and maximum levels above 1000 µg/kg (Ezkiel *et al*., 2012; Tangendjaja *et al*., 2008). In contrast, FUMB1 were the most predominant mycotoxins with relatively high concentrations in most of the analyzed samples, especially in corn and compound feed (Table 2). Co-occurrence of *Fusarium* mycotoxins, including FUMs, DON, and ZEA, is common in cereals and particularly high concentrations were reported in corn (Asam *et al*., 2017; Kovalsky *et al*., 2016; Kamala *et al*., 2015). In addition, resistance of these mycotoxins to processing conditions (Kovalsky *et al*., 2016). Makes corn-based feed prone to contamination with them, as is illustrated in this study showing that the highest mean concentration of FUMB1, DON and ZEA in compound feed corresponded roughly to their counterparts in the ingredients (Table 2).

Overall, the results show that none of the samples analyzed herein exceeded the most stringent maximum tolerable limits (MTLs) of 20, 2000, 5000 and 20,000 µg/kg for AFB1, ZEN, DON, and FUMB1, respectively(http://www.mycotoxins.info/en/regulations) enforced in the EU for poultry feeds, suggesting that these mycotoxins, taken individually, do not raise concern regarding their safety for poultry. It should be emphasized, however, that there is a lack of data on the overall toxicity resulting from multi-mycotoxin contamination of feeds, which may be enhanced by synergistic effects, as was demonstrated e.g., between aflatoxins and fumonisins in rats (Qian *et al*., 2016) and by epidemiologically (Kamala *et al*., 2018). However, given the low levels of AFs in all samples, this is unlikely to be of a paramount concern in our case regarding the toxicity of aflatoxins. The low aflatoxin contamination of all analyzed samples is a reassuring indication, as this highly potent mycotoxin raises the most concern to the safety of foods and feeds. Also, high levels of AFB1 in feeds were reported to reduce the quality and productivity of poultry products and to be carried over to eggs and meat used in human diet (Sarma *et al*., 2017). Therefore, the results obtained in this study provide an indication of the acceptable sanitary quality of the analyzed Moroccan poultry feed and feed-ingredients. This can also reflect the quality of the imported ingredients from the countries of origin, since crop contamination with mycotoxins usually begins in the field and continue in the following post-harvest stages. The application of good manufacture practices (GMPs) in the Moroccan poultry feed producing factories may as well account for such results. Nonetheless, some concern may still arise from the possible synergistic action between the other mycotoxins including the *Fusarium* mycotoxins (Sarma *et al*., 2017). detected herein, which may, upon chronic exposure, produce various adverse health effects on poultry and/or affect the farm productivity (Kovalsky *et al*., 2016; Kubena *et al*., 1997). Albeit, this is unlikely in view of the short life span of poultry before slaughtering.

Comparison of the two analytical methods ELISA and HPLCMS/MS

It is well established that HPLC-MS/MS is currently one of the most reliable and efficient techniques for mycotoxin quantification; however, its high cost and the need for skilled operators to carry out analyses hinders its routine use in the field to act in a real time in case of problems (Benkerroum *et al*., 2019). Clean-up and matrix preparation and internal standards to fit-for-purpose are particular tedious and may lead to erroneous results if improperly conducted (Krska *et al*., 2017). On the contrary, ELISA technique offers a practical, quick, easy, and relatively cost-effective means to monitor mycotoxins in the field. Therefore, we analyzed 10, 5, and 5 samples of corn, soya and wheat, respectively, taken randomly for the determination of AFB1, DON, FUMB1 and ZEA concentrations with both ELISA and HPLC-MS/MS techniques for comparison purposes. Close results were only obtained for FUMB1, while discrepancies between the concentrations AFB1 and DON were too high to be attributed to experimental errors (Table 3). Given the well-established superior performances of HPLC-MS/MS in terms of specificity, sensitivity, and accuracy compared to ELISA, our results suggest that ELISA may still be useful as an in-field techniques for screening, although it was not appropriate for complete feed.

Table 3: Mean concentrations (mg /kg) of mycotoxins in 10, 5, 5 samples of corn, soya and wheat and also in 10 sample of the poultry feed formulation containing this ingredient, as determined by ELISA and HPLC MS/MS methods

Commodity	AFB1		DON		FUMs		ZEA	
	ELISA	HPLC MS/SM	ELISA	HPLC MS/SM	ELISA	HPLC MS/SM	ELISA	HPLC MS/SM
Corn	2,88	0,35	3213	2523	6241	6542	-	49,9
Soya	-	-	-	6,61	26	74,0	-	75,7
Wheat	-	-	2,80	3158	45	179	-	3,98
Compound feed	-	0,73	-	3420	-	4072	-	59,0

- *Not detected*
(for all samples analyzed, the OTA is note detected)

CONCLUSION

Poultry products (meat and eggs) are increasingly contributing to economy and food security as valuable and affordable source of animal proteins worldwide. However, their safety and wholesomeness depend largely on the feed provided. Mycotoxin contamination of poultry feed is of increased concern due to its impact on the productivity and health of poults, which requires sustained monitoring of increasingly high number of mycotoxins in feed and feed-ingredients. Aflatoxins and *Fusarium* mycotoxins (e.g., ZEA, FUMs, DON, and NIV) are the most relevant, as they have long been frequently associated with grains used as the main ingredients in feed formulations. This study consisted of an analysis of three main grains (corn, soya, and wheat) used in poultry feed formulations in Morocco as well as in compound feed. The obtained results show that the concentrations of the major mycotoxins of concern to poultry health were below the EU regulatory limits, suggesting that they do not raise a real concern if taken individually. AFs known for their deleterious effects on poultry and for being transmissible by carry-over to the foods derived thereof were detected in particularly low concentrations in corn and compound feed, and they were not detected in soya and wheat samples. Yet, safety concerns can not be completely ruled out due to possible synergistic effect between the different mycotoxins present in the studied commodities. Also, the analysis was carried out for a relatively low number of samples of each product, and during the dry season in Morocco when the humidity in unfavorable for the growth and mycotoxin production by toxigenic fungi under good storage conditions. Therefore, the study should continue to perform the analyses during different seasons of the year, mainly the rainy season of December through March and on a larger number of samples.

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