Enzyme immunoassay for monitoring aflatoxins in eggs

UNIVERSITÀ DEGLI STUDI DI TORINO

ENZYME IMMUNOASSAY FOR MONITORING AFLATOXINS IN EGGS

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ABSTRACT

Rapid and sensitive competitive enzymatic immunoassays for measuring most relevant aflatoxins in eggs have been developed by synthesizing two hapten derivatives. Polyclonal antibodies raised against a hapten obtained from aflatoxin B1 (AFB1) were exploited to set an AFB1-selective assay, whereas antibodies obtained through immunising with a hapten derived from aflatoxin M1 (AFM1) allowed us to detect four principal aflatoxins (B1, G1, B2, and G2) and the most relevant AFB1 metabolite (AFM1) with detection limits in eggs of 0.3 µg kg^{-1} for AFB1, AFG1, and AFM1 and 3 µg kg^{-1} for AFB2 and AFG2, respectively. We also established a rapid and simple protocol for extracting aflatoxins from eggs by employing aqueous methanol (70%) followed by partitioning with hexane to remove fats. The whole analytical process is simple, very rapid (the extraction requires 14 minutes, and the assay is completed in 30 minutes) and proved to be accurate and precise enough (recoveries ranged from 84 to 100% and RSD% were within 20% for intra- and inter-assay experiments) to be proposed as a first level screening method for the monitoring of the occurrence of aflatoxins in egg.

KEYWORDS: Aflatoxin M1, group-selective immunoassay, aflatoxin extraction

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INTRODUCTION

Aflatoxins are secondary metabolites produced by moulds of the *Aspergillus* family, which contaminate several crops, including cereals, oilseeds, tree nuts, and spices. Due to the fact that *Aspergillus* moulds could grow on crops pre-, during, and post-harvest and that their toxic metabolites are very stable to chemical and physical stresses, aflatoxins have been found in raw and processed materials and represent the most common cause of chemical contamination of foodstuffs, according to the European Union alert system (EU Rapid Alert System for Food and Feed). Among about 300 different natural aflatoxins, the most diffuse and toxic is the aflatoxin B1 (AFB1). It is produced by *A.flavus* and *A. parasiticus* and has been recognized as the most potent carcinogen for human (International Agency for Research on Cancer, 2002). Besides AFB1, principal aflatoxins are: aflatoxin B2 (AFB2), which is produced by the same mould as AFB1 but in a lesser extent; aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2), which belonged to *A. parasiticus* (AFG1 is the predominant toxin excreted by *A. parasiticus*). Maximum acceptable levels for AFB1 and for the sum of all the four aflatoxins have been set worldwide in commodities susceptible to contamination and intended for human consumption (European Commission, 2010) or for feeding farm animals (European Commission, 2003). Furthermore, it has been demonstrated that dairy cattle, sheep and goats fed with AFB1 contaminated feedstuffs transfer the aflatoxin to milk partially as the unmodified precursor, but primarily as a hydroxylated metabolic product (Van Egmond, 1989). This AFB1 metabolite excreted to milk (aflatoxin M1, AFM1) retains most of AFB1 toxicity (Caloni, 2006) (International Agency for Research on Cancer, 2002); therefore, maximum tolerable levels have been established also for AFM1 in milk (European Commission, 2010). Conversely, the carry-over of AFB1 into meat of animals fed with contaminated material is controversial (Hayes, 1977) (Díaz-Zaragoza, 2014) (Hussain, 2010) and the risk for consumers associated to meat consumption seems to be negligible. Recently, the potential AFB1 carry-over into eggs in laying hens fed with contaminated crops has been investigated. Pandey and Chauhan reported on the effect of ingesting AFB1 contaminated grain on chicks (Chauhan, 2007). AFB1 residues were detected in eggs and breast muscle of AFB1-fed hens. The carry-over of AFB1 was confirmed by the works of Hassan et al (Hassan, 2012) and of Herzallah (Herzallah, 2013) who also studied the combined effect of the four major aflatoxins. He found an analogous carry-over for AFB1 and the other three aflatoxins. Hassan

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et al observed that AFB1 residues appeared in eggs after 5 days from starting administration of contaminated feedstuffs and that AFB1 accumulated in eggs with increasing amounts found for protracted feeding with contaminated grain. Nevertheless, the amounts of aflatoxin residues found in eggs were very low in every case and varied between 0.01% (Herzallah, 2013) and 0.07% (Hassan, 2012) of the aflatoxin intake. This result could be partially explained by the fact that AFB1 is metabolized by the bird (Rawal, 2010). Indeed, the metabolic transformation of AFB1 was responsible of diseases observed on hens and highlighted by the same authors. However, none of the preceding papers considered the metabolic detoxification pattern which led to the formation of hydroxylated metabolites of AFB1 (AFM1 and aflatoxin Q1) (Rawal, 2010) and authors did not investigate the occurrence of AFM1 in eggs, similarly to what is done in milk.

World egg production involved over 60 millions tonnes per year from a total of approximately 6.5 billion hens and expanded by more than two per cent a year in the last decades (Nutriad). China is the world largest egg producer and is accounted for one third of the entire world production, followed by USA and India. Countries belonging to the European Union produce approximately 7.5 million tonnes of egg per year (European Commission). The demand of feed for sustain poultry production makes suspect on its quality, also because most of the ingredients used to produce poultry feed are used for human consumption. Thus, the risk that materials discarded for human consumption could be employed as feedstuffs is not negligible. Furthermore, since poultry production is relatively inexpensive and widely available and, as poultry meat and eggs are considered low-cost sources of protein, their production is strongly encouraged in developing countries, which led sometimes to not adequate housing and management of animals and feedstuffs and to increased risk of contamination (FAO, 2013).

Therefore, the accessibility of rapid, cost-effective, and simple methods of analysis to detect aflatoxins in eggs would help scientists to better investigate the occurrence of these contaminants and to more adequately support conclusions on risks for human health due to consumption of eggs belonging to hens fed with aflatoxin contaminated materials. Moreover, it would allow the efficient and continuous monitoring of such contaminants to assure food security. Analytical methods to determine aflatoxins in eggs currently available are based on chromatographic techniques coupled to fluorescence or mass

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spectrometric detection (Herzallah, 2009) (Garrido Frenich, 2011) (Capriotti, 2012). However, to ensure the rapid and cost-effective screening of large numbers of sample and the availability of analytical methods applicable in developing countries, the exploitation of immunochemical methods of analysis, which are known to address requirements of rapidity, simplicity and inexpensiveness, is advisable.

This study aimed at developing a rapid and sensitive competitive enzymatic immunoassay for measuring most relevant aflatoxins in eggs. Therefore, two hapten derivatives were synthesized, with the objective of raising polyclonal antibodies able to bind the principal aflatoxin (AFB1), the main AFB1 metabolic product (AFM1) and possibly the other three relevant aflatoxins (AFG1, AFB2, and AFG2). By exploiting those antibodies, two direct competitive immunoassays could be proposed: an AFB1-selective assay and a group-selective assay. This last allowed us to detect all above-mentioned mycotoxins. Moreover, aflatoxin extraction from eggs was optimized with the aim of fulfilling the same requirements of rapidity, easy operation and cost-effectiveness to allow the applicability of the whole analytical protocol as a screening method in routinary monitoring of aflatoxin contamination in eggs.

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MATERIALS AND METHODS

Materials

Aflatoxin B1, aflatoxin M1, aflatoxin B2, aflatoxin G1, aflatoxin G2, ochratoxin A (OTA), deoxynivalenol (DON), fumonisin B1 (FB1), and zearalenone (ZEA) standard solutions were Oekanal certified solutions from Sigma Aldrich (St. Louis, MO, USA). Aflatoxin B1 and aflatoxin M1 powders were purchased from Fermentek (Jerusalem, Israel). Bovine serum albumin (BSA), N,N'-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), and 3,3'5,5'-tetramethylbenzidine liquid substrate (TMB) were purchased from Sigma- Aldrich (St. Louis, MO, USA). Horse-radish peroxidase (HRP) was purchased from Roche Diagnostics (Milan, Italy). Sephadex G-25 cartridges were from GE Healthcare (Milan, Italy). Dimethylformamide (DMF), methanol (HPLC grade) and all other chemicals and microtitre plates were obtained from VWR International (Milan, Italy).

Production of the hapten, hapten-protein conjugates and antibodies

Aflatoxin B1-O-(carboxymethyl)oxime (AFB1-cmo) and Aflatoxin M1-O-(carboxymethyl)oxime (AFM1-cmo), were synthesized from AFB1 and AFM1, respectively, as previously reported (Chu, 1977). The two haptens (Figure 1) were conjugated to BSA by the DCC/NHS ester method and used for immunization; AFB1-cmo was also conjugated to HRP to generate the labelled probe. Briefly, equimolar amounts of AFB1-cmo or AFM1-cmo, DCC and NHS were dissolved in anhydrous DMF and the mixture was incubated at 4°C temperature for 2 hours. Proper amounts of the mixture were then added to protein solutions prepared in 0.13 M $NaHCO_3$, to obtain a final molar ratio of 200:1 (AFB1-cmo or AFM1-cmo:BSA), and 10:1 (AFB1-cmo:HRP). BSA conjugates (AFB1-BSA and AFM1-BSA) were incubated overnight at room temperature, while the HRP conjugate (AFB1-HRP) was reacted for 1 hour at room temperature. Separation of conjugates from by-products and excess of reagents was carried out by gel filtration on a Sephadex G-25 cartridge (mobile phase: phosphate buffer saline).

Anti-AFB1 and anti-AFM1 antibodies were produced by Davids Biotechnologie (Germany) by using their standard immunization protocol for rabbit polyclonal antibodies (Davids Biotechnologie) and sera were collected after 70 days from the first injection. The immunoglobulin fraction was obtained from antisera by ammonium sulphate precipitation and used without further purification.

Competitive direct ELISA

We prepared the immunoreactive solid phase by coating wells with 150 µl of anti-AFB1 or anti-AFM1 rabbit polyclonal antibodies diluted in carbonate/bicarbonate buffer pH 9.6 (overnight at 4°C). To assure complete saturation of well surface, after washing plates with 0.05% Tween 20, we further incubated 300 µl of phosphate buffer supplied with 0.15M NaCl and 0.5% BSA (PBS@BSA) for 1 hour at room temperature, followed by washing wells with 0.05% Tween 20.

The construction of calibration curves involved mixing 100 µl of AFB1-HRP (0.08 µg ml^{-1}) in PBST@BSA and 100 µl of AFB1 standards diluted in aqueous methanol (35%) at concentrations ranging from 0 to 2500 µg l^{-1}. After 15 minute incubation in immunoreactive wells, unbound reagents were removed by five washings with a washing solution including 0.3M NaCl and 0.05% Tween 20. Colour development was obtained by a 15 min incubation with TMB (200 µl per well). The addition of 50 µl of sulphuric acid (2M) stopped colour

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development and allowed absorbance recording at 450 nm. For egg samples, extracts prepared as described below were directly added to wells instead of AFB1 standards. All standards were measured in duplicate, whereas samples were measured in quadruplicate.

Unknown sample concentrations were determined by interpolation on the calibration curve, where the signal was plotted against the log of analyte concentration. For each experiment, a calibration curve was determined by a nonlinear regression analysis of the data using the four-parameter logistic equation.

Cross-reactivity study

We prepared calibration curves for several mycotoxins, by employing the same protocol described above, except from the concentration range used, which depended on the investigated mycotoxin. We used 0 - 2.5 μg l^{-1} for AFM1; 0 - 10 μg l^{-1} for AFG1, AFB2, and AFG2; and 0 - 100 μg l^{-1} for OTA, DON, FB1, and ZEA, respectively.

Relative cross-reactivity was calculated as follows:

CR% = (IC_{50} AFB1 / IC_{50} mycotoxin)*100

where IC_{50} is the mycotoxin concentration which cause 50% inhibition of the maximum observed signal.

The estimated limit of detection for aflatoxin (except from AFB1) was derived from CR% by dividing LOD calculated for AFB1 by the cross-reactivity as follows:

Estimated mycotoxin LOD = (LOD AFB1 / CR% mycotoxin)*100

Samples and sample preparation

Egg samples were purchased in large stores (Large-scale distribution, LSdis) or directly in farms of small-scale producers (Farm) of the North-West of Italy during the period January-March 2014.

Two samples that did not show any detectable residues of aflatoxins were taken as the blank for the optimization of the extraction protocol and for recovery experiments. Fortified samples were prepared by adding 0.5, 2.0, and 10.0 μg kg^{-1} or 1.0, 4.0, and 10.0 μg kg^{-1} of AFB1, respectively, to the egg before performing the extraction.

Egg yolk was manually separated and gently mixed before extractions. To optimize extraction of aflatoxins from egg we weighed 1 g of the homogenized sample and mixed it with 5 ml of various extraction media: 1) water to which 0.05% Tween 20 had been added (0.05% Tween 20); 2) water : methanol 30:70 (methanol 70%); 3) water : methanol 70:30, in which 0.3M NaCl had been added to water (methanol 70%/0.3M NaCl); and 4) water : methanol 70:30 followed by the addition of hexane to remove fatty components. After 2 minutes of vigorous stirring at room temperature, samples were centrifuged at 3200 x g to reduce foam and to remove denatured proteins. Supernatants were diluted 1+1 with water and analysed by the direct competitive ELISA. Each sub-sample was extracted in duplicate and analysed in quadruplicate.

The optimal protocol involved extraction with aqueous methanol (70%) followed by defatting with hexane. Briefly, we recovered the supernatant after the extraction described above and added 1 ml of hexane. We vigorously stirred the mixture for 2 minutes again, separated the upper organic layer by centrifugation (5 minutes at 3200 x g), and diluted the underlying layer 1+1 with water before submitting it to analysis.

RESULTS AND DISCUSSION

Competitive direct ELISAs

Four antisera were produced by immunizing two rabbits with AFB1-BSA and two with AFM1-BSA, respectively. These were tested by a non-competitive indirect ELISA, by following the protocol described in the experimental section, except for the facts that antibodies were coated at increasing dilutions and that the analyte was absent. All four generated antisera show high titres. Their performance in competitive conditions were also tested by determining the rate between the signal obtain from each antiserum diluted at its IC_{50} value in the presence of AFB1 (10 µg l^{-1}) and in the absence of AFB1 (0 µg l^{-1}). Both anti-AFB1 antisera and one of the anti-AFM1 antisera exhibited high binding properties towards the analyte (AFB1). One anti-AFB1 antiserum and the anti-AFM1 with high binding properties were used for the study.

Competition experiments were carried out under various combinations of antibody dilutions, AFB1-HRP concentrations and times of incubation to set a sensitive and rapid assay. Since aflatoxin extraction from food materials involves the use of aqueous methanol in most cases (Stroka, 1999) (Reiter, 2009), the effect

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of the solvent on assay performance was also evaluated. We found that methanol interference was negligible for amounts up to 20%, thus we established to dilute AFB1 standards in 35% methanol for calibration. In this way, extracts, which would contain 70% methanol, could easily match calibrators by being diluted 1+1 with water. In the meantime, standard and extracts would be further diluted 1+1 in wells by mixing them with the AFB1-HRP solution which permitted us to reach overall methanol content below 20% during the assay.

Preliminary, we optimized two systems: a homologous assay, which employed the anti-AFB1 antibody and the AFB1-HRP as the probe, and a heterologous assay, which employed the anti-AFM1 antibody and the same AFB1-HRP probe, since heterology is known to promote greater sensitivity (Holthues, 2005) (Z. Wang, 2013). Figures of merits of the two optimized assays, carried out as described in the experimental section, are summarized in Table 1 and typical inhibition curves obtained under optimized conditions for the two systems are shown in Figure 3. Both systems were highly sensitive and very rapid, provided that results could be achieved in 30 min. Assay characteristics are comparable in terms of detectability and dynamic range.

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Selectivity

Since we did not know if the supposed ingested aflatoxin would be preserved as it or metabolized by the bird before transference to the egg, we aimed at developing an assay which would cross-react with the four major aflatoxins and also with the metabolic product, aflatoxin M1.

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We studied the selectivity of the two developed ELISAs by measuring their cross-reactivity towards mentioned aflatoxins (AFB1, AFM1, AFG1, AFB2, and AFG2) and towards other mycotoxins, whit chemical structures completely unrelated to those of aflatoxins (DON, FB1, OTA, and ZEA). The interference with both assays was negligible for all unrelated mycotoxins. These did not cause any inhibition of the binding between antibodies and the AFB1-HRP probe at levels up to 100 µg l^{-1}.

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Instead, we observed a dissimilar binding capacity of the two ELISAs when aflatoxins were applied, which was imputable to the antibodies used. The system based on the anti-AFB1 antiserum showed a selective pattern, in which only AFG1 and AFB2 were detected in some extent, besides AFB1 (Figure 4). Otherwise,

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the assay based on the anti-AFM1 antiserum behaved as a non-selective system, since AFB1, AFM1 and AFG1 inhibited the binding of AFB1-HRP probe to the antibodies quite similarly, and also AFB2 and AFG2 were effective at competing with the probe for antibody binding (Figure 3b and 4). Therefore, we assumed the assay based on the anti-AFB1 antiserum as applicable for the selective determination of AFB1 (AFB1-assay), whereas the assay exploiting the anti-AFM1 antiserum was considered as being able to detect most aflatoxins and thus as a group-selective assay. Since the purpose of the work, the system based on anti-AFM1 antiserum was further characterized and applied to aflatoxin determination in eggs.

AFB1 extraction from egg

According to the literature, the most popular protocols for extracting aflatoxins from food and feed to be measured by immunoenzymatic methods involve aqueous methanol with percentages varying from 60 to 80% and typically 70% (Stroka, 1999) (Reiter, 2009). Depending on the sample nature, high amounts of sodium chloride and/or a step aimed at removing fats could be introduced (Garden, 2001) (Shadbad, 2012). However, also aqueous extractants have been reported to be effective in the extraction of aflatoxins from cereals (Maragos, 2008) (Anfossi, 2011). Therefore, we compared recovery of AFB1 from an artificially contaminated egg sample, when extracted by various media including: water, aqueous methanol (70%), aqueous methanol with sodium chloride added, and aqueous methanol followed by defatting with hexane. Results of tests on the egg fortified at three AFB1 levels: 0.5 (low), 2.0 (medium), and 10.0 µg kg^{-1} (high) are summarised in Figure 5. The aqueous extraction medium was inadequate (recoveries comprises between 15 and 24%) while 70% methanol confirmed its superior quality. The addition of the salt slightly impaired performance, while appending a step aimed at removing fats from extracts contributed to achieve quantitative results, most likely because fats interfered in the immunoassay determining underestimation.

Analytical validation of the group-selective immunoassay for measuring aflatoxins in eggs

The LOD of the method was calculated by interpolation on the AFB1 standard curve as the analyte concentration corresponding to the mean signal of the zero standard (obtained by averaging the signal of eight replicate sets) minus three times its standard deviation and it was 0.03 µg l^{-1} for both the AFB1-assay

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and the group-selective assay (Table 1). Adjusted for dilution due to extraction, the limits of detection of the two formats compared to egg samples were 0.3 µg kg^{-1} for AFB1. The group-selective assay allowed us to measure AFG1 and AFM1 at the same level as AFB1 (LOD 0.3 µg kg^{-1}) and AFB2 and AFG2 with a LOD of 3 µg kg^{-1}, which are in the range of limits imposed by the European legislation on various food, except for AFM1 in milk (European Commission, 2010).

To evaluate the accuracy of the method, two egg samples were fortified with AFB1 (concentrations of 1.0, 4.0, and 10 µg kg^{-1}), extracted and analysed by the group-selective assay. Results are summarized in Table 2. Recovery values ranged from 84 to 100%, thus indicating a good accuracy of the assay when applied to egg samples. The precision of the method was determined by extracting and analysing replicates of artificially contaminated egg samples, which were fortified with AFB1 at three levels: 0.5, 2.0, and 10.0 µg kg^{-1}. The assay was carried out in eight replicates on the day for the evaluation of within-assay precision and on four different days for the evaluation of the between-assay precision (Table 3). The values of RSD% were calculated at each nominal concentration level and ranged from 8 to 20%, which fulfilled FDA requirements for the validation of bioanalytical methods according to FDA guidance (Anfossi, 2009). These results proved that the developed assay is suitable as a first level screening method for the detection of aflatoxins in eggs, with good accuracy and precision.

Finally, we collected a total of 50 samples, belonging to the large distribution and to small farms located in the North West of Italy, during the period of January-March 2014. No positive samples were found in the market according to analysis through the developed group-selective ELISA method.

CONCLUSIONS

The occurrence of aflatoxin contamination in cereals poses severe risk to consumers not only through their consumption, but also through entering the food chain. This has been widely demonstrated in the case of milk belonging to dairy animals fed with aflatoxin contaminated crops. Aflatoxin carry-over in eggs has also been reported recently. Therefore, the development of analytical methods aimed at measuring such contaminants in eggs would help assuring food safety within the whole food chain. For this purpose, we developed an enzymatic assay for monitoring aflatoxins in eggs and established a fast, simple and effective

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extraction protocol, based on the use of aqueous methanol as the extractant and of hexane to remove fatty components of the matrix.

The developed assay is sensitive, also because sample extracts need a very limited dilution before being analysed, thus LOD in eggs are 0.3 µg kg^{-1} for principal aflatoxins (AFB1, AFG1, and AFM1) and 3 µg kg^{-1} for AFB2 and AFG2. In addition, the method is simple, very rapid (the extraction requires 14 minutes, and the assay is completed in 30 minutes), and we could decide to measure the most relevant aflatoxin by the AFB1-selective assay or, alternatively, to detect generically chemical structures structurally related to AFB1 through the group-selective assay. The last allowed us to determine several aflatoxins and could potentially apply for the detection of AFB1, AFG1, AFG2 and AFG2 contaminated samples due to carry-over, as for the detection of samples containing the most relevant AFB1 metabolite (AFM1).

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FIGURE CAPTIONS

Figure 1: Structure of the five principal aflatoxins, the hapten derivatives used for immunization and for preparation of the enzymatic tracer, and the mycotoxins used in the cross-reactivity study.

Figure 2: Characterization of polyclonal antibodies generated by immunizing two rabbits with AFB1-BSA (anti-AFB1-R1 and anti-AFB1-R2) and two with AFM1-BSA (anti-AFM1-R1 and anti-AFM1-R2). Signals recorded by the competitive direct ELISA when AFB1 was added at 0 (black column) and 10 µg l^{-1} (grey column).

Figure 3: Typical inhibition curves obtained under optimized conditions for the two ELISA systems developed: a. AFB1 standard curve in the selective assay (signal vs. AFB1 concentration); b. standard curves for the five major aflatoxins in the groupe-selective assay (○ AFB1, △ AFM1, □ AFG1, ▽AFB2, ◇AFG2). For comparison, the B/B0 vs. mycotoxin concentration is shown, where B is the signal observed for the mycotoxin concentration and B0 is the signal of the blank.

Figure 4: Cross-reactivity of aflatoxins determined by the two ELISAs towards major aflatoxins.

Figure 5: Extraction of AFB1 from an artificially contaminated egg sample by varying the extraction medium. Egg sample was fortified at three AFB1 concentration levels: 0.5 µg l^{-1} (low, black), 2 µg l^{-1} (medium, grey), and 10 µg l^{-1} (high, white).

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