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# Excretion pattern of aflatoxin $M_1$ in milk of goats fed a single dose of aflatoxin $B_1$

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## ABSTRACT

The feedstuffs used in dairy animals must be able to give consumers confidence about the wholesomeness of milk with regard to aflatoxin contamination. The aim of this study was to determine the excretion patterns of aflatoxin $M_1$ (AFM1) in the milk of dairy goats fed a single dose of pure aflatoxin $B_1$ (AFB1), which can occasionally occur if feeds are infected by hot-spot growth of molds that produce aflatoxins. Five dairy goats in midlactation were administered 0.8 mg of AFB1 orally. Individual milk samples were collected for 84 h after AFB1 dosage. Aflatoxin $M_1$ was found in milk in the highest concentration. In all goats, AFM1 was not detected in milk before AFB1 administration, but was detected in the first milking following AFB1 administration. The excretion pattern of AFM1 concentration in milk was very similar in all goats even if the values of the concentration differed between animals. The peak values for AFM1 concentration in milk was observed in milk collected during the milking at 3 and 6 h. After the peak, the AFM1 in milk disappeared with a trend that fitted well a monoexponential decreasing function, and the toxin was not detected after 84 h. Only about 0.17% of the amount of AFB1 administered was detected as AFM1 in milk, and about 50% of this was excreted in the first liter of milk yielded after AFB1 intake. Correct procedures to prevent growth of molds, and consequent AFB1 contamination, on the feedstuffs for lactating goats represent the key to providing consumers a guarantee that milk is not contaminated by AFM1.

**Key words:** aflatoxin $B_1$, aflatoxin $M_1$ excretion, goat milk

## INTRODUCTION

Aflatoxins represent a relevant group of mycotoxins produced by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*. These molds principally invade plant tissue, in particular when damaged, and mainly produce aflatoxin $B_1$ (**AFB1**). This toxin is included in group 1 as a human carcinogen by the International Agency for Research on Cancer (Lyon, France; IARC, 1993) because it has been associated with hepatocellular carcinoma in humans. More risk for the occurrence of AFB1 is commonly associated with foodstuffs produced in warm climates (Cotty and Jaime-Garcia, 2007), and the contamination can occur before or after harvest mainly on starchy cereal crops, cottonseeds, and peanuts (Richard, 2007). Evaluation of real exposure of farm animals to mycotoxins is not always easy because the contaminants may not be distributed uniformly in feeds and their assortment of diet composition. This implies that adequate sampling procedures are needed to obtain the maximum certainty on the contamination level in the commodity (Whitaker et al., 2010).

Aflatoxin occurrence in feedstuffs is particularly relevant where there is on-farm feed storage and the climate is high in temperature and humidity. In those conditions it is possible that molds will grow in spots, giving isolated pockets of feed contamination by the aflatoxins. In a bin of corn infected by the growth of *Aspergillus* during warm weather, Shotwell et al. (1975) found large variability in the mycotoxin contamination: in kernels collected in the hot spot pocket of stored grain, the concentration of aflatoxins was about 23,000 to 38,000 μg/kg, whereas the toxin was not detected in many other parts of the bin where the mold did not develop.

Since the 1960s, the milk of cows fed a diet contaminated by AFB1 has been reported to contain a toxicant with a toxic effect like that of aflatoxins (Allcroft and Carnaghan, 1963). This hydroxylated metabolite of AFB1 was isolated (de Iongh et al., 1964) and named by Holzapfel et al. (1966) as aflatoxin $M_1$ (**AFM1**). Aflatoxin $M_1$, initially classified by IARC as a Group 2B human carcinogen (IARC, 1993), is now included in the category of agents carcinogenic to humans, so now it is in the Group 1 human category (IARC, 2002).

To reduce human and animal exposure to toxic effects of aflatoxins, many countries regulate the maximum level of aflatoxins that can occur in feeds for dairy animals

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and the level of AFM1 in milk. However, those limits vary extensively between countries. In European Union countries, the maximum levels of AFB1 in complete feed for dairy animals is 0.005 mg/kg (European Commission, 2003), whereas in the United States, the action levels for total aflatoxins in animal feeds and ingredients for dairy animals is 0.020 mg/kg (US FDA, 1994).

The relevance of milk in human consumption, especially children, induced the European Community to evaluate the presence of AFM1 in milk and dairy products. The European Commission (2001) has established that maximum levels of AFM1 in liquid milk and dried or processed milk products should not exceed 50 ng/kg. After this regulation, it was amended by setting the limit at 25 ng/kg for AFM1 in infant formulas and follow-on formulas, including milk (European Commission, 2004). The US Food and Drug Administration has established an action level of 0.50 μg/kg in whole, low-fat, and skim milk (US FDA, 1996).

During the last decades several studies have been carried out to quantify the relationship between AFB1 intake and the resulting AFM1 contamination of milk in several dairy livestock species. Most of these experiments were focused on knowing the extent of the transfer of AFM1 in the milk of goats (Smith et al., 1994; Rao and Chopra, 2001), cows (Veldman et al., 1992; Diaz et al., 2004; Masoero et al., 2007), and sheep (Battacone et al., 2003, 2005, 2009) fed a contaminated diet for a long time. Some studies have been conducted on the transfer of AFM1 into milk as a result of a single consumption of AFB1 in sheep (Battacone et al., 2003) and cows (Trucksess et al., 1983). From a practical standpoint, the use of highly contaminated feed by dairy farmers is unlikely; however, a single accidental feeding of contaminated feed may happen and can lead to milk AFM1 content above tolerance levels (Battacone et al., 2003).

Because the occasional contamination of feedstuffs can occur even in dairy goat farms, it is of great importance to thoroughly understand the dynamic of excretion pattern of the toxin in the milk and its carryover to formulate an accurate risk analysis. For this reason, an experiment was designed to simulate an accidental ingestion of aflatoxin by lactating goats by using a single-dose intake of AFB1 and then measuring the evolution of AFM1 excretion in milk and its relationship with the secretion of the main milk components (i.e., lactose, protein, and fat).

## MATERIALS AND METHODS

### Animals

The experiment was conducted in spring in accordance with the guidelines of the European Communities Council Directive (November 24, 1986; 86/609/EEC). Five multiparous goats of Saanen breed in mid-lactation (average 120 DIM and 59.0 ± 3.28 kg of BW; mean ± SD) were used. The daily milk yield averaged 3.35 ± 0.47 kg and (mean ± SD). The does, each of which was named with a letter from A to E, were kept in a shed, fed 1.5 kg/d of a commercial concentrate and hay grass, and had ad libitum access to water. Before the experiment, goats were milked twice per day (every 12 h).

On the first day of the experimental period, the mammary gland of each goat was completely emptied by stripping by hand, after an intravenous injection of 1 IU of oxytocin (Sigma-Aldrich S.r.l., Milan, Italy) just before toxin administration. Then, each goat received 0.8 mg of AFB1 in a single dose of pure AFB1, corresponding to about 0.0136 mg/kg of BW. The amount of AFB1 administered in this experiment was higher than the supposed quantity of toxin that can be consumed by dairy goats that intake feed contaminated near the tolerance level in the United States (20 μg/kg) or European Union (5 μg/kg). However, the dose of 0.8 mg of AFB1 is about equivalent to the aflatoxin content in 25 to 35 g of highly contaminated (23,000–38,000 μg/kg) kernels, such as in hot spots in buckets of corn stored in improper conditions (Shotwell et al., 1975). The AFB1 dose was obtained by dissolving the pure toxin (A-6636; Sigma Chemical Co., St. Louis, MO) in methanol, and a pellet of concentrate was used as the aflatoxin carrier. The administration of AFB1 was performed by placement of a contaminated pellet directly into the oral cavity of each goat.

The goats were milked using a milking machine for 84 h following AFB1 administration. Individual milk yield was recorded and milk samples were collected at 0, 1, 3, 6, and 12 h, and every 12 h thereafter until 84 h after dosing. An aliquot of each milk sample was promptly analyzed for fat, protein (N × 6.38), and lactose, and another aliquot was stored at −18°C until the AFM1 content could be analyzed.

### Analytical Procedures

Milk was analyzed for fat, protein, and lactose with a MilkoScan 4000 apparatus (Foss Electric A/S, Hillerød, Denmark). The extraction of AFM1 from milk was done using an immunoaffinity technique and the concentration was determined by HPLC as described by Battacone et al. (2005). Briefly, defatted milk was passed through an immunoaffinity column (Afla M1; Vicam LP, Watertown, MA); then, the AFM1 was eluted with methanol and evaporated to dryness under nitrogen. The resultant residue was dissolved in acetonitrile/water (25:75 vol:vol) and 1% acetic acid

and injected into the HPLC apparatus. The AFM1 was separated on a Hewlett-Packard 1100 HPLC chromatograph (Hewlett-Packard Co., Palo Alto, CA) connected to a reversed-phase C18 column (Zorbax SB, 5-μm particle size, 150 × 4.6 mm i.d.; Agilent Technologies Inc., Palo Alto, CA) equipped with a Hewlett-Packard 1100 fluorescence detector. The eluent was a solution of acetonitrile/water (25:75 vol:vol) and 1% acetic acid, at a flow rate of 1 mL/min. Quantitation of AFM1 was carried out using the calibration curve obtained by plotting the peak area for each standard against the quantity of AFM1 injected. The limit of detection value for AFM1 was 4 ng/L.

### Calculations

The milk AFM1 concentration versus time (h) curves obtained after the AFB1 administration were determined separately for each goat. The milk secretion rate (g/h) was calculated by dividing the yield of milk (g) of each milking by the corresponding milking interval, assuming that the milk secretion rate was constant over this period. Lactose, protein, and fat secretion rates (g/h) and AFM1 excretion rate (ng/h) were calculated by multiplying their concentrations in milk from each milking by the milk secretion rate in the relative period. In those calculations, it was assumed that secretion and excretion was constant during that period. The percentage of AFB1 excreted in milk as AFM1 was calculated as the ratio between the total output of AFM1 in milk (sum of AFM1 excreted at each milking) and the total AFB1 administered, separately for each goat.

### Statistical Analyses

Statistical analysis of the data was performed using SAS/STAT software (SAS Institute Inc., Cary, NC). Each goat was considered the experimental unit and milk yield, AFM1, and milk components (fat, protein, and lactose) were analyzed with a mixed model that included the fixed effect of time (h) after AFB1 administration and the random effect of goat. Pairwise comparisons among different times were performed using the Tukey test.

The decreasing pattern of AFM1 excretion in milk after peak (AFM1 in ng/L) was studied by adapting the data to the following exponential function: $\text{AFM1} = a \exp(bt)$, where $a$ is the intercept, $b$ is the exponential parameter, and $t$ is the time from the administration of the toxin in hours, by using the nonlinear procedure of SAS.

To study the relationship between the cumulative percentages (**CPerc**) of AFM1 excreted in the milk and the cumulated milk yield (MY, in liters) after AFB1 administration, the monomolecular kinetic equation $\text{CPerc of AFM1} = a[1 - \exp(-b\text{MY})]$ was fitted to the data by using the nonlinear procedure of SAS. Linear correlations among hourly secretion rate of milk, lactose, protein, fat, and the excretion rate of AFM1 in milk were tested by the Pearson procedure.

## RESULTS AND DISCUSSION

During the whole experimental period, none of the goats showed any evident illness or health disturbance related to AFB1 administration.

Aflatoxin $M_1$ was not detected in the milk sampled before the goats' AFB1 exposure, but it was found in all milk samples collected 1 h after the toxin administration (Figure 1). In 3 goats, the peak AFM1 concentration was reached around 3 h after toxin administration, whereas in 2 goats, the peak was reached around 6 h. After the peak, the AFM1 concentration decreased rapidly in the milk of all goats. These values were slightly higher than the corresponding limit of detection value in milk that was collected after 72 h, and the toxin was not detected in the samples at 84 h. Although the shape of the excretion curves was similar among animals, the single trend shows a broad variability in the ascending tracts.

The presence of AFM1 in milk collected with the first milking after AFB1 administration is in accordance with the results of a previous experiment carried out on lactating ewes fed a single dose of pure AFB1 (Battacone et al., 2003). Even if the dose of AFB1 administered to sheep was higher (2 mg/head) than in the current experiment, the animals weighed 43.5 ± 6.57 kg of BW (mean ± SD) and, moreover, the first milking was 6 h after the AFB1 dosage. Furthermore, in large ruminants, Gallo et al. (2008) showed that just 5 min after the administration of an oral bolus containing 4.9

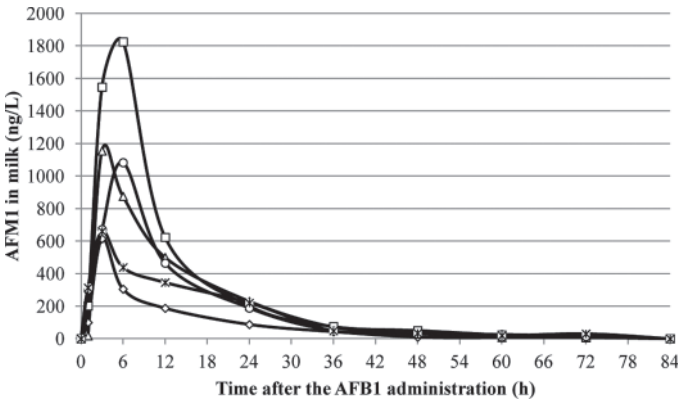


**Figure 1.** Individual excretion pattern of aflatoxin $M_1$ (AFM1) in the milk of goats fed a single oral dose of aflatoxin $B_1$ (AFB1).

mg of AFB1, not only the same toxin, but also its hydroxy derivative AFM1, were detectable in the plasma. Previously, but with higher doses, Cook et al. (1986) have reported the appearance of AFM1 in the blood of steers 30 min after a single dosage of 0.2 to 0.8 mg/kg of BW of a mix of aflatoxins (about 75% of AFB1).

All of those results indicate that the absorption of the AFB1 in the gastrointestinal tract of adult ruminants is very fast, as is its hydroxylation to AFM1 and release in the blood. The short interval between AFB1 administration and the detection of its metabolite in the milk observed in our goats confirms that the absorption of the toxin is already relevant in the rumen as hypothesized by Cook et al. (1986). Furthermore, Upadhaya et al. (2009) demonstrated that the extent of degradation of AFB1 in rumen fluid in goats is influenced by the type of feed.

Studies of Larsson et al. (1989, 1994, 2003) and Larsson and Tjälve (1996) on cattle, sheep, horses, and swine have evidenced that, in addition to the liver, microsomes from the mucosa located in the upper respiratory and alimentary pathways also have a high capacity to activate AFB1. Furthermore, in some cases, the pathway of AFB1 activation in mucosal tissues have AFM1 as one of the dominating metabolites (Larsson et al., 1989). However, these aspects should not have affected our results because in this experiment, the pellet containing the AFB1 was introduced in the distal portion of the oral cavity to guarantee its immediate transit to the esophagus.

The high variability observed for AFM1 concentration in milk during the first 6 h after the administration, previously reported by other authors (Goto and Hsieh, 1985; Rao and Chopra, 2001), seems to be the result of the differences in several steps that affect the transfer of the AFM1 into the milk, such as uptake of the AFB1 in the gastrointestinal tract, extent of bioconversion of AFB1 into AFM1 in the liver and in the other tissues, and the amount of AFBI or AFM1 excreted in the urine and feces, which also are the major routes of elimination of these toxins in lactating goats (Helferich et al., 1986).

The average AFM1 concentration in milk (Figure 2) was significantly higher at 3 and 6 h after the administration with respect to the others times ($P < 0.01$), confirming our previous findings in dairy ewes (Battacone et al., 2003). The disappearance of AFM1 in milk was very fast; only traces of the toxin were detectable at 72 h. The decrease in AFM1 concentration after the peak was faster than reported in ewes (Battacone at al., 2003) as a consequence of the higher peak value found in this experiment. The value of the contamination level of milk moved below the European Union limit (0.05 μg/kg) after 36 h; furthermore, the time at which

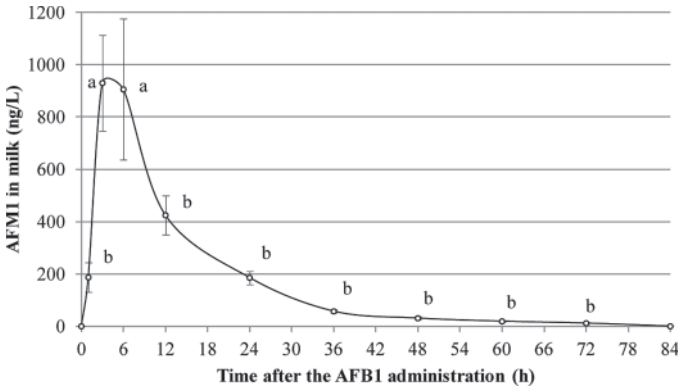


**Figure 2.** Average excretion pattern of aflatoxin $M_1$ (AFM1) in the milk of goats fed a single oral dose of aflatoxin $B_1$ (AFB1). Each data point represents the mean (± SE) of the 5 animals. Different letters (a and b) indicate differences ($P < 0.05$) between sampling hours.

AFM1 was no longer detectable in milk was the same for all animals and was not related to the concentration of AFM1 at the peak.

The equation fitting the data of milk AFM1 disappearance from the peak is

$$\text{AFM1 (ng/L)} = 1{,}265.68 \times \exp(-0.08\ t);$$
$$\text{pseudo-R}^2 = 0.799.$$

where $t$ is the time from the administration of the toxin (expressed in hours) and pseudo-$R^2$ is calculated as $1 - SS_{residual}/SS_{total\ corrected}$ (where SS is the sum of squares). Both the intercept (a) and exponential parameter (b) of the equation are significantly different from zero at the 95% confidence level (CI: $944 < a < 1{,}587$; $-0.12 < b < -0.04$).

The decreasing pattern (Figure 3) of the toxin excretion in milk is surprisingly regular, in contrast with the oscillatory trend previously observed in dairy ewes (Battacone et al., 2003) and in other species, both in

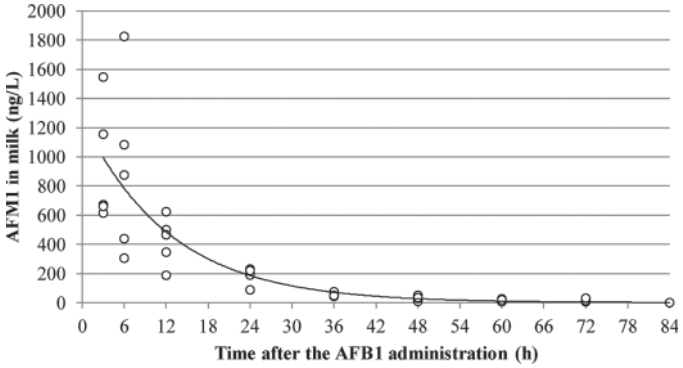


**Figure 3.** Disappearance pattern of aflatoxin $M_1$ (AFM1) in the milk of goats fed a single oral dose of aflatoxin $B_1$ (AFB1).

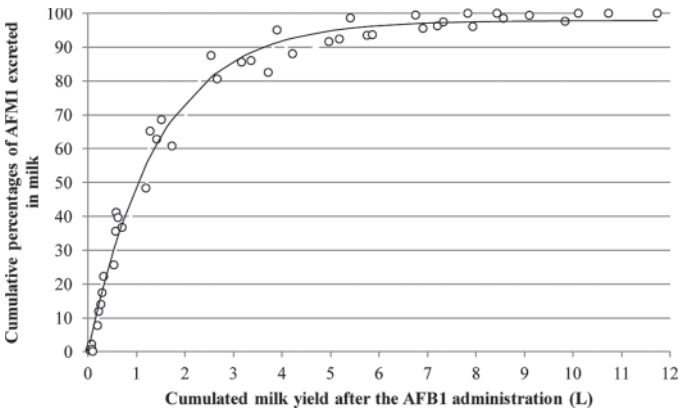


**Figure 4.** Cumulative percentages of aflatoxin $M_1$ (AFM1) excreted in the cumulated milk yield of goats fed a single oral dose of aflatoxin $B_1$ (AFB1).

blood (Cook et al., 1986) and milk (Nabney et al., 1967; Trucksess et al., 1983; Goto and Hsieh, 1985). This is probably due to the higher single dose of toxin administered to animals than that used in this experiment. These oscillatory responses suggest a significant contribution of the reabsorption/redistribution of toxin via enterohepatic circulation when its concentration in the blood is very high, as suggested by Trucksess et al. (1983) in their experiment with cows. The relevance of the reabsorption and redistribution via enterohepatic circulation in the excretion of the mycotoxin in ruminants is well described by Xiao et al. (1991) in sheep fed a single dose of another mycotoxin, such as ocratoxin A.

The average of the total amount of AFM1 excreted in milk was 0.17% (± 0.06, ± SE) of the AFB1 that we administered to the animals. This value is slightly lower than that found by Helferich et al. (1986) in goats treated with ring-labeled [$^{14}$C]-AFB1 (0.18–0.38%), but higher than what we reported (0.032%) in dairy ewes (Battacone et al., 2003).

The following equation fits the data of the cumulative percentages of AFM1, excreted in milk, in function of the cumulated milk yield (Figure 4):

$$\text{CPerc of AFM1} = 97.82\ [1 - \exp(-0.695 \times \text{MY})];$$

$$\text{pseudo-R}^2 = 0.997.$$

Both the intercept (a) and exponential parameter (b) of the equation are significantly different from zero at the 95% confidence level (CI: $95.87 < a < 99.78$; $-0.751 < b < -0.639$). About 50% of the total AFM1 excreted was in the first liter of milk yielded by each goat and about 90% of the toxin was excreted in the first 3.8 L.

The mean excretion rate of the AFM1 per hour in milk had a pattern similar to that of its concentration in the milk (data not shown). The increase in the AFM1 excretion rate during the first 6 h following the administration may indicate that, in this time, the incoming flow of the toxin in the blood was higher than its flow out. The analysis of correlations between the secretion rate of milk, lactose, protein, and fat and the excretion rate of AFM1 in milk (Table 1) showed that the AFM1 excretion rate was not significantly correlated with any other rate. This confirmed that the passage of the AFM1 from the blood into the alveolar lumens in the mammary gland was not affected by the activities of secretory cells and, moreover, represented a further clue for AFM1 passive diffusion across the mammary gland epithelium.

The results of this experiment indicate that a sporadic intake of AFB1 may cause serious consequences for the transfer of AFM1 into milk. However, the dangerous contamination of milk is a transient event that affects the milk yielded during the first 36 to 48 h after AFB1 intake.

## CONCLUSIONS

Although the feeds commonly used in dairy goat diets seem not to be at a very high risk for high aflatoxin contamination, sporadic relatively high ingestion of AFB1 can occur. However, this occurrence could be relevant in light of the consequent level of milk contamination by AFM1. The main results of this experiment showed that 1) AFM1 was detected in the milk of all goats 1 h after the AFB1-containing pellet was administered; 2) the level of AFM1 in goat milk initially increased, and the peak of concentration was reached in milk collected after 3 to 6 h; 3) the pattern of milk AFM1 disappearance after the peak was well fitted by a monoexponential decreasing function and the toxin was not detected after 84 h; 4) the amount of AFM1 excreted in

**Table 1.** Pearson correlation coefficients (*P*-value in parentheses) among hourly secretion rates of milk, lactose, protein, and fat and hourly excretion rate of aflatoxin $M_1$ (AFM1)

| Item | AFM1 | Lactose | Protein | Fat |
|---|---|---|---|---|
| Lactose | −0.18 (0.239) | | | |
| Protein | −0.08 (0.608) | 0.94 (<0.001) | | |
| Fat | −0.10 (0.501) | 0.60 (<0.001) | 0.65 (<0.001) | |
| Milk | −0.17 (0.256) | 0.98 (<0.001) | 0.90 (<0.001) | 0.55 (<0.001) |

goat milk was about 0.17% of the single dose of AFB1 administered; and 5) about 50% of the AFM1 excreted in milk was found in the first liter yielded after AFB1 intake (i.e., within 12 h). Even if the negative risk of AFM1 in milk appears temporarily limited, the correct procedures to prevent growth of molds, and consequent AFB1 contamination, on the feedstuffs for lactating animals represent the key to satisfying the consumers. Those procedures should also be implemented in on-farm feed production and storage to avoid the hot-spot growth of aflatoxigenic mold, which might be difficult to detect, as it is confined to a small area.

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